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Title

Phylogenomics Supports the Monophyly of Aphelids and Fungi and Identifies New Molecular Synapomorphies

Authors:

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Running title: Phylogenomics and Megasytematics of Holomycota

24 *Abstract.*- The supergroup Holomycota, composed of Fungi and several related lineages of
25 unicellular organisms (Nucleariida, Rozellida, Microsporidia, and Aphelida), represents one of
26 the major branches in the phylogeny of eukaryotes. Nevertheless, except for the well-
27 established position of Nucleariida as the first holomycotan branch to diverge, the relationships
28 among the other lineages have so far remained unresolved largely owing to the lack of
29 molecular data for some groups. This was notably the case aphelids, a poorly known group of
30 endobiotic phagotrophic protists that feed on algae with cellulose walls. The first molecular
31 phylogenies including aphelids supported their sister relationship with Rozellida and
32 Microsporidia which, collectively, formed a new group called Opisthosporidia (the
33 ‘Opisthosporidia hypothesis’). However, recent phylogenomic analyses including massive
34 sequence data from two aphelid genera, *Paraphelidium* and *Amoeboaphelidium*, suggested that
35 the aphelids are sister to fungi (the ‘Aphelida+Fungi hypothesis’). Should this position be
36 confirmed, aphelids would be key to understanding the early evolution of Holomycota and the
37 origin of Fungi. Here, we carry out phylogenomic analyses with an expanded taxonomic
38 sampling for aphelids after sequencing the transcriptomes of two species of the genus
39 *Aphelidium* (*A. insulamus* and *A. tribonematis*) in order to test these competing hypotheses.
40 Our new phylogenomic analyses including species from the three known aphelid genera
41 strongly rejected the Opisthosporidia hypothesis. Furthermore, comparative genomic analyses
42 further supported the Aphelida+Fungi hypothesis via the identification of 19 orthologous genes
43 exclusively shared by these two lineages. Seven of them originated from ancient horizontal
44 gene transfer events predating the aphelid-fungal split and the remaining 12 likely evolved *de*
45 *novo*, constituting additional molecular synapomorphies for this clade. Ancestral trait
46 reconstruction based on our well-resolved phylogeny of Holomycota suggests that the
47 progenitor of both fungi and rozellids, was aphelid-like, having an amoeboflagellate state and
48 likely preying endobiotically on cellulose-containing, cell-walled organisms. Two lineages,
49 which we propose to call Phytophagea and Opisthophagea, evolved from this ancestor.
50 Phytophagea, grouping aphelids and classical fungi, mainly specialized in endobiotic predation
51 of algal cells. Fungi emerged from this lineage after losing phagotrophy in favour of
52 osmotrophy. Opisthophagea, grouping rozellids and Microsporidia, became parasites, mostly
53 of chitin-containing hosts. This lineage entered a progressive reductive process that resulted in
54 a unique lifestyle, especially in the highly derived Microsporidia.

55

56 *Keywords:* Fungi, Aphelida, Holomycota, phylogenomics, horizontal gene transfer,
57 synapomorphy

58 The Aphelida is a diverse group of endobiotic phagotrophic unicellular protists that feed on
59 algae that have cellulose walls (green algae and stramenopiles) (Gromov 2000; Karpov et al.
60 2014, 2017). Despite being known since the end of the 19th century (Zopf 1885), aphelids were
61 neglected until recent years when, thanks to classical culturing efforts, they gained attention
62 due to their idiosyncratic cell biology and phylogenetic position within the Holomycota (one
63 of the two main branches of the Opisthokonta) (Karpov et al. 2014; Adl et al. 2019), resulting
64 in a significant increase in their known biodiversity (Karpov et al. 2020).

65 The morphology of the aphelid zoospores, which can be flagellated and/or ameboid, has
66 been widely used for species classification (Karpov et al. 2019; Letcher and Powell 2019).
67 From the ~20 described species, only 11 freshwater species belonging to the genera
68 *Aphelidium*, *Paraphelidium* and *Amoeboaphelidium* have been molecularly characterized by
69 SSU rRNA gene sequencing (Letcher and Powell 2019; Karpov et al. 2020). The fourth
70 described genus, represented by the marine species *Pseudaphelidium drebesii*, has not been
71 reported since its original description (Schweikert and Schnepf 1996, 1997) and sequence data
72 is missing. SSU rRNA gene phylogenies showed that aphelids belong to the Holomycota,
73 together with nucleariids, rozellids, microsporidia, and fungi (fungi defined here as the
74 monophyletic group of holomycotan species that excludes all the previous taxa) (Karpov et al.
75 2013, 2014, 2017; Letcher et al. 2015). These phylogenies, also including environmental
76 sequences from diverse habitats, determined that the aphelids are monophyletic with moderate
77 to high bootstrap support (72-100%) (Simon et al. 2015; Karpov et al. 2020; Seto et al. 2020).
78 Within the Holomycota, some of these phylogenies grouped, albeit with weak support, aphelids
79 with *Rozella* (Letcher and Powell 2018) and Microsporidia (Vávra and Lukeš 2013) in a clade
80 sister to fungi that has been named Opisthosporidia (Karpov et al. 2014).

81 The phylogenetic signal of SSU rDNA is too limited to resolve ancient speciation events in
82 eukaryote evolution, not only at supergroup level (Adl et al. 2019) but also within clades,
83 including opisthokont phyla (e.g. Galindo et al. 2019). Transcriptome and genome sequencing
84 has facilitated multi-marker phylogenetic analysis that is critical for resolving the phylogeny
85 of all eukaryotes (Sibbald and Archibald 2017; Lax et al. 2018). Recently, phylogenomic
86 analyses using three datasets derived from transcriptome data of the aphelid *Paraphelidium*
87 *tribonematis* supported the monophyly of this aphelid and fungi, rendering the Opisthosporidia
88 clade paraphyletic (Torruella et al. 2018). Later, an independent phylogenomic study including
89 markers from two *Amoeboaphelidium* genomes again supported the monophyly of aphelids
90 and fungi (Tikhonenkov et al. 2020). However, these studies included limited taxon sampling,

91 so the monophyly of aphelids and the sister relationship between the aphelids and the fungi
92 requires further validation.

93 To test the two competing phylogenetic hypotheses, “Opisthosporidia” versus
94 “Aphelida+Fungi”, we sequenced transcriptomes of two species from a third aphelid genus,
95 *Aphelidium* (*A. insulamus* and *A. tribonematis*) (Karpov et al. 2016, 2020) and we used
96 phylogenomic methods to analyze all of the available aphelid genomes and transcriptomes,
97 together with representatives of all of the known Holomycota lineages. We resolved the
98 monophyly of these three aphelid genera and confirmed the position of aphelids as the sister
99 lineage to fungi, thereby rejecting the Opisthosporidia hypothesis. We also found several
100 molecular synapomorphies that further support the Aphelida+Fungi hypothesis, including
101 shared genes ancestrally obtained by horizontal gene transfer (HGT) and genes likely evolved
102 *de novo*. Based on these results, we propose a reorganization of the internal systematics of the
103 Holomycota with the creation of two new major taxa, the Phytophagea, containing Aphelida
104 and Fungi, and the Opisthophagea, containing Microsporidia and Rozellida.

105

106

MATERIALS AND METHODS

107

Culturing, RNA Extraction, Transcriptome Sequencing, Assembly and Decontamination

108

109 *Aphelidium insulamus* O-14 (X-134) (Karpov et al. 2020) and *Aphelidium tribonematis* P-2
110 (X-102) (Karpov et al. 2016) cultures were maintained with their prey, the xantophyte alga
111 *Tribonema gayanum* (Strain 20 CALU), in mineral medium BG11 at 15°C with a 12h-12h
112 night-day cycle. The cultures were initially cleaned from other contaminant eukaryotes by
113 picking zoospores of both aphelid species by micromanipulation and transferring them into
114 pure *T. gayanum* cultures.

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For each species, total RNA was extracted from three cultures at different growing stages,
8, 16 and 30 days after being transferred to healthy *T. gayanum* cultures. These times
corresponded to peaks in the abundance of different aphelid cell cycle stages (cysts, trophonts,
and zoospores) and were used to maximize the gene coverage of the transcriptomes (see
Torruella et al. 2018). The grown cultures were filtered together through 0.22 µm pore-
diameter filters (Millipore) using a vacuum filtration pump. Filters were incubated in 2 ml
Eppendorf tubes filled with lysis buffer from the RNeasy mini Kit (Qiagen), following the
manufacturer protocol. Extracted RNA was quantified with a Qubit fluorometer (ThermoFisher
Scientific). One cDNA Illumina library was constructed for each species after polyA mRNA
selection and paired-end (2 × 125 bp) sequenced with Illumina HiSeq 2500 chemistry v4
(Eurofins Genomics, Germany). We obtained 9,261,168 reads for *A. tribonematis* P-2 and

125 7,271,713 reads for *A. insulamus* O-14. Raw read sequences have been submitted to GenBank
126 under BioProject accession numbers PRJNA719988 (*A. insulamus* O-14) and PRJNA720686
127 (*A. tribonematis* P-2).

128 Paired-end read maximum quality scores were checked with FastQC v0.11.8 (Andrews
129 2010), and each transcriptome was *de novo* assembled using rnaSpades v3.15.0 (Bankevich et
130 al. 2012) with default parameters. Predicted proteins were obtained using Transdecoder v2
131 (<http://transdecoder.github.io>) with default parameters and Cd-hit v4.6 (Li and Godzik 2006)
132 with 100% identity. Decontamination of host transcripts was done using the *T. gayanum*
133 transcriptome as local Blastp (Altschul et al. 1990) query to exclude identical sequences, as
134 previously done by Torruella et al. (2018). To assess genome completeness, we used BUSCO
135 v2.0.1 on the decontaminated predicted proteomes with the eukaryota_odb9 dataset of 303
136 near-universal single-copy orthologs (Simão et al. 2015).

137

138 *Phylogenomic Analyses*

139 The dataset of 351 conserved proteins from Lax et al. (2018) was updated by Blastp search
140 of each protein against the inferred proteomes of representatives of all known major eukaryotic
141 lineages, enriched in opisthokonts but also in stramenopiles to ensure the elimination of *T.*
142 *gayanum* contaminants. Each protein marker was aligned with MAFFT v7 (Katoh and Standley
143 2013) and trimmed with TrimAl with the automated1 option (Capella-Gutiérrez et al. 2009).
144 Alignments were manually inspected and edited with AliView (Larsson 2014) and Geneious
145 v6.06 (Kearse et al. 2012). Single-protein trees were reconstructed with IQ-TREE v1.6.11
146 (Nguyen et al. 2015) with the corresponding best-fitting model. Each single-protein tree was
147 manually inspected to discard contaminants and possible cases of horizontal gene transfer or
148 hidden paralogy. At the end of this curation process, we kept a final taxon sampling of 25
149 species, including Nucleariida, Aphelida, Rozellida, Microsporidia, and Fungi (Table S1,
150 Supplementary Material available on Dryad), and 175 protein markers which were present in
151 all aphelid species (including the species with the least amount of available data,
152 *Amoeboaphelidium protococcarum*). All proteins were realigned, trimmed and concatenated
153 using Alvert.py from the package Barrel-o-Monkeys
154 (<http://rogerlab.biochemistryandmolecularbiology.dal.ca/Software/Software.htm>), creating a
155 final supermatrix with 59,889 amino acids. For details on the origin of the sequence data see
156 Table S4 (Supplementary Material available on Dryad).

157 Bayesian inference (BI) analyses were done with PhyloBayes-MPI v1.5a (Lartillot et al.
158 2009) on CIPRES Science Gateway (Miller et al. 2010) with both CAT-Poisson and CAT-GTR

159 models (Lartillot and Philippe 2004), with two MCMC chains and run for 10,000 generations,
160 saving one every 10 trees. Analyses were stopped once convergence thresholds calculated
161 using bpcomp were reached (i.e. maximum discrepancy <0.1 and minimum effective size
162 >100) and consensus trees were constructed after a burn-in of 25%. Maximum Likelihood
163 (ML) analyses were done with IQ-TREE v1.6.11 (Nguyen et al. 2015) with the best-fitting
164 model (LG+F+R6) identified by ModelFinder (Kalyaanamoorthy et al. 2017). Additional ML
165 trees were done using the PMSF model (Wang et al. 2018) to account for among-site rate
166 heterogeneity and with the C60 profile mixture model. ML statistical support was calculated
167 with 1000 ultrafast bootstraps (Minh et al. 2013). All trees were visualised and edited with
168 FigTree v1.4.3 (Rambaut 2016).

169 In addition to the phylogenetic analysis of concatenated protein markers, we examined the
170 175 ML phylogenetic trees of individual proteins (inferred with RAxML v.8.1.6 with the
171 PROT+CAT+LG+F model and 100 bootstrap replicates (Stamatakis 2014)) using a coalescent
172 approach with ASTRAL-III under the multi-species coalescent model with default parameters
173 (Zhang et al. 2018). To minimize possible systematic bias in our concatenated dataset due to
174 the presence of fast-evolving sites, known to be more prone to homoplasy and compositional
175 bias (Rodríguez-Ezpeleta et al. 2007), we reconstructed a series of trees by progressively
176 removing the fastest-evolving sites, 5% of sites at a time. For that, among-site substitution rates
177 were inferred using IQ-TREE under the -wsr option and the best-fitting model for a total of 19
178 new data subsets (Table S2, Supplementary Material available on Dryad). We then
179 reconstructed phylogenetic trees for all these subsets using IQ-TREE with the same best-fitting
180 model as for the whole concatenated dataset.

181

182 *Detection of Molecular Synapomorphies*

183 We inspected 23 opisthokont proteomes (including the three aphelids *P. tribonematis*, *A.*
184 *tribonematis* and *A. insulamus*) to look for proteins that were unique to aphelids and fungi. We
185 first carried out clustering of orthologous genes with OrthoFinder v1.1.20 (Emms and Kelly
186 2015) with default parameters. We then identified the orthologs shared by aphelids, chytrids,
187 blastocladiomycetes and the rest of fungal representatives. Representative protein sequences
188 of each candidate ortholog were blasted against the non-redundant GenBank database using
189 Blastp (Altschul et al. 1990) with and without Fungi (taxid:4751). The first 100 hits of both
190 searches were downloaded and merged into single-protein datasets together with positive hits
191 from our 23 opisthokonts. The putative identity and function of each ortholog was assigned
192 using domain searches both in Blastp and HMMER using hmmscan (<http://hmmer.org/>). Cd-

193 hit v4.6 with 100% identity was used to eliminate redundant sequences from the datasets. To
194 verify that the Blast searches did not miss distant homologs of the orthologs found only in
195 aphelids and fungi, we reconstructed HMM profiles for each of those orthologs using the
196 hmmbuild program from the HMMer package v3.2.1 (Mistry et al. 2013). These profiles were
197 used to scan the GenBank RefSeq database with the hmmsearch program from the same
198 package. The taxonomy (NCBI taxID identifiers) of the retrieved hits was obtained using the
199 protein accession number as reference to query the NCBI taxonomy database
200 (<https://www.ncbi.nlm.nih.gov/taxonomy>). The hits for each ortholog and their taxonomy were
201 sorted by e-value and printed in tabular output files.

202 Each protein dataset was aligned using MAFFT v7 (Katoh et al. 2002) and trimmed with
203 TrimAl in automated1 mode. Single-protein ML trees were inferred using IQ-TREE (Nguyen
204 et al. 2015) with the best-fitting model selected with the IQ-TREE TESTNEW algorithm as
205 per BIC. All datasets and trees were checked manually for possible contaminating sequences.

206 Assembled transcriptomes, predicted proteins, HMM profiles and hmmsearch results,
207 phylogenomic datasets, and molecular synapomorphies have been deposited in figshare at
208 https://figshare.com/projects/Aphelida_Extended_Data/111539 and in the Dryad Digital
209 Repository: <https://doi.org/10.5061/dryad.j3tx95xdn>.

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211

Ancestral State Reconstruction

212 We inferred ancestral states for the following five traits in various clades within the
213 Holomycota: flagellum (presence / absence), pseudopodia (presence / absence), feeding mode
214 (osmotrophy / phagotrophy), preferred food/host (decaying organic matter / bacteria / chitinous
215 organisms / plants or algae), and habitat (freshwater or soil / marine). We used the Bayesian
216 inference phylogenetic tree of 25 species as reference phylogeny (see above) and two
217 approaches for ancestral state reconstruction implemented in Mesquite 3.70 (Maddison and
218 Maddison 2021): a parsimony approach with the unordered states model of evolution for
219 categorical characters, and a likelihood approach with the Markov k-state 1 parameter (Mk1)
220 model.

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222

RESULTS AND DISCUSSION

223

The Aphelid Clade is the Sister Lineage to Fungi

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226

After sequencing, assembly and decontamination of the two aphelid transcriptomes, we
obtained 45,543 transcripts for *A. insulamus* O-14 and 49,586 transcripts for *A. tribonematis*
P-2. Comparison with the BUSCO eukaryotic dataset of 303 near-universal single-copy

227 orthologs (Simão et al. 2015) indicated very high transcriptome completeness for the two
228 species (97,7% for *A. insulamus* O-14; 96,7% for *A. tribonematis* P-2). We used 175 conserved
229 protein markers after updating a previous dataset (Lax et al. 2018) to include the aphelid
230 sequences and representatives of major holomycotan groups for a final taxon sampling of 25
231 species (Table S1, Supplementary Material available on Dryad) and 59,889 amino acid
232 positions. Both maximum likelihood (ML) and Bayesian inference (BI) phylogenetic analyses
233 of this data set supported the overall same tree topology (Fig. 1A). Nucleariida emerged as the
234 first branch among Holomycota, followed by two sister clades, one containing *Rozella* and
235 Microsporidia, in agreement with previous analyses (Brown et al. 2009; Liu et al. 2009;
236 Galindo et al. 2019), and another containing Aphelida and Fungi. Within fungi, despite their
237 reduced taxon sampling included in this tree, we found support for the “chytrid first” hypothesis
238 already recovered in recent phylogenomic studies (Spatafora et al. 2016; Mikhailov et al. 2017;
239 Tedersoo et al. 2018; Galindo et al. 2021). Both ML and BI methods yielded maximum support
240 (bootstrap (BS) 100% and posterior probability (PP) 1, respectively) for the monophyly of
241 aphelids and their placement as sister lineage to fungi (Fig. 1A and Supplementary Fig. S1a-d,
242 Supplementary Material available on Dryad). These analyses confirmed with a richer taxon
243 sampling and stronger support the Aphelida+Fungi hypothesis previously proposed by
244 Torruella et al. (2018). We tested the possible influence of fast-evolving sites on this result by
245 applying a slow-fast approach (Brinkmann and Philippe 1999) to progressively remove the
246 fastest-evolving sites (in 5% steps, see Fig. 1B and Table S2, Supplementary Material available
247 on Dryad). The monophyly of aphelids+fungi obtained maximum support (BS >99%) in all
248 steps until only 35% of the sites remained and the phylogenetic signal was too scarce to resolve
249 any deep-level relationship (Fig. 1B). In fact, the monophyly of aphelids+fungi received higher
250 statistical support than the monophyly of fungi. On the contrary, the monophyly of
251 Opisthosporidia (aphelids+rozellids+microsporidia) was never well supported. These results
252 were confirmed by the combined analysis of the 175 ML trees based on individual protein
253 markers with a multi-species coalescent model (Zhang et al. 2018). The resulting species tree
254 had a similar topology as the BI and ML trees based on the concatenation of these markers and
255 showed full support for the Aphelida+Fungi hypothesis (Fig. 1C and Supplementary Fig. S1e,
256 Supplementary Material available on Dryad).

257 Despite their overall congruence, the ML and BI trees based on the concatenated dataset
258 showed conflicting topologies for the internal relationship of aphelids. The two
259 *Amoeboaphelidium* species were paraphyletic in all phylogenies, as observed in Tikhonenkov
260 et al. (2020), with *A. occidentale* sister to *P. tribonematis* (e.g., Fig. 1A), whereas *A.*

261 *protococcarum* was the first aphelid branch to diverge in ML trees (with BS between 85 and
262 94%, Supplementary Fig. S1c-d, Supplementary Material available on Dryad) as well as in the
263 coalescent-based species tree (Supplementary Fig. S1e, Supplementary Material available on
264 Dryad). In the BI trees, *Aphelidium* spp. were the earliest diverging aphelids with maximum
265 support under both CAT-Poisson and CAT-GTR models (Supplementary Fig. S1a-b,
266 Supplementary Material available on Dryad).

267

268

Molecular Synapomorphies of Aphelids and Fungi

269 Given the phylogenetic evidence for the monophyly of aphelids and fungi, we looked for
270 proteins unique to these two groups but not present in other eukaryotes. These unique proteins
271 could be considered molecular synapomorphies and would therefore provide further support
272 for their monophyly. We identified a total of 80,441 orthogroups in our dataset of opisthokont
273 proteomes. 93 of these orthologs were shared by at least two species of aphelids, chytrids,
274 blastoclads and the rest of fungal representatives. We then verified if these 93 orthologs were
275 absent in other eukaryotic groups by Blast against the non-redundant GenBank database. 74
276 orthologs were present in other eukaryotes and 19 were found only in aphelids and fungi. We
277 further confirmed the absence of these 19 orthologs in other eukaryotes using a more sensitive
278 HMM-based search against the NCBI Reference Sequence (RefSeq) database. The 19
279 orthologs found only in aphelids and fungi were likely already present in the last common
280 ancestor of both groups. To test this idea, the sequences of these orthologs were aligned,
281 trimmed and used for ML tree reconstruction (Table S3 and Supplementary Figs. S2-S20,
282 Supplementary Material available on Dryad).

283

284 After manual checking of these trees, we identified 7 genes that appeared to have been
285 acquired by HGT from bacteria and 12 that likely evolved *de novo* in a common ancestor of
286 aphelids and fungi. Among the 7 putative HGT cases, all but one ortholog (OG0000718) had
287 functional domains assigned (Table S3, Supplementary Material available on Dryad). The
288 remaining 12 proteins did not have homologs in other organisms and, therefore, we considered
289 them as *de novo* evolved proteins, although the possibility that they were also acquired by HGT
290 from not yet sequenced prokaryotic or eukaryotic donors cannot be discarded. Two of these
291 proteins (OG0007603 and OG0011744) did not contain any known domain (Supplementary
292 Figs. S17 and S20, Supplementary Material available on Dryad), and another two (OG0007040
293 and OG0009707) carried the conserved domains of unknown function (DUF) DUF2418 and
DUF2423, respectively (Supplementary Figs. S14 and S19, Supplementary Material available

294 on Dryad). For the other nine proteins, we could infer a putative function based on their domain
295 composition (Table S3, Supplementary Material available on Dryad).

296

297 The seven proteins derived from ancestral HGT events in the aphelids+fungi clade had
298 various putative functions and cellular localizations, from intramembrane mechanosensitive
299 channels (OG0001250) to cytoskeletal components (OG0002101) and extracellular enzymes
300 (OG0002372) (Fig. 2 and Supplementary Figs. S3, S4, and S6, Supplementary Material
301 available on Dryad). One interesting case was that of OG0003785, a GTP cyclohydrolase II
302 enzyme (GTPCH2) involved in riboflavin synthesis. GTPCH2 has been described only in fungi
303 (mainly yeasts), bacteria and plants (Oltmanns et al. 1969; Bereswill et al. 1998; Herz et al.
304 2000; Spoonamore et al. 2006; Yadav and Karthikeyan 2015). However, our analysis of the
305 OG0003785 ortholog (Supplementary Fig. S7, Supplementary Material available on Dryad)
306 showed that the plant sequences are extremely divergent (Supplementary Fig. S7B,
307 Supplementary Material available on Dryad) and only their C-terminal half is homologous to
308 the fungal and bacterial sequences. By contrast, the GTPCH2 proteins from aphelids and fungi
309 are highly similar to the bacterial ones along the whole protein length. Thus, the gene encoding
310 GTPCH2 (*RIB1* or *RibA*) was most likely acquired from bacteria by an ancestor of aphelids
311 and fungi, which gained this function independently from plants. GTPCH2 catalyzes the first
312 step of riboflavin biosynthesis by converting GTP into 2,5-diamino-6-(5-phospho-D-
313 ribosylamino)-pyrimidin-4(3H)-one (DARP), then DARP is processed by several enzymes to
314 generate the final product (Anam et al. 2020). It has been shown that GTPCH2 activity is
315 required for *RIB1*-dependent protection of yeast cells from nitrosative stress (Anam et al.
316 2020). This stress is caused by excessive levels of nitric oxide (NO) leading to cellular damage
317 or death, as it has been demonstrated in yeast (Almeida et al. 2007; Yoshikawa et al. 2016).
318 Defence against NO is essential also for pathogenicity, as shown in *Candida albicans*, which
319 uses flavohemoglobin to weaken the toxicity of NO released by the host (Hromatka et al. 2005;
320 Zakikhany et al. 2007; Martin et al. 2011; Liao et al. 2016). Riboflavin biosynthesis is essential
321 in microbes but absent in humans and therefore this pathway is an ideal antimicrobial drug
322 target with minimum host toxicity (Yadav and Karthikeyan 2015). The horizontal transfer of
323 this gene from bacteria to an ancestor of aphelids and fungi could have played an important
324 role in the evolution of pathogenicity in this eukaryotic clade.

325 In addition to these shared ancestral HGTs in aphelids and fungi, we found 12 genes that
326 appear to have evolved *de novo* in their ancestor. They encoded proteins with various putative
327 cellular localizations, including the nucleus (e.g., OG0007045) and mitochondrion (e.g.,

328 OG0008189) (Fig. 2, and Supplementary Figs. S15 and S18, Supplementary Material available
329 on Dryad). Among them, it is worth highlighting OG0006536 (Supplementary Fig. S11,
330 Supplementary Material available on Dryad), which corresponds to a S25-like mitochondrial
331 ribosomal protein from the 37S small ribosomal subunit (RSM25-like). Mitoribosomes are
332 attached to the mitochondrial inner membrane and, in yeast, consist of a small (37S) and a large
333 (54S) subunit. The 37S subunit contains at least 33 different proteins and one rRNA (15S)
334 (Saveanu et al. 2001; Pfeffer et al. 2015; Desai et al. 2017). Similarly to some other yeast
335 mitoribosomal proteins (Saveanu et al. 2001), the RSM25-like protein had no known homolog
336 in any other eukaryotic or prokaryotic lineage, making it a conspicuous synapomorphy for the
337 aphelids+fungi group.

338 The ortholog OG0007007 (Supplementary Fig. S12, Supplementary Material available on
339 Dryad) provided another interesting synapomorphy. It corresponds to the AAA-ATPase Vps4-
340 associated protein 1 (Vfa1). Controlling and remodelling membranes (e.g., extracellular vesicle
341 formation, retroviral budding, cell abscission during cytokinesis, multivesicular body
342 formation) is a vital process for cellular homeostasis (Oliveira et al. 2013; Vild and Xu 2014).
343 It is especially important in fungi, since two of their canonical traits, apical growth and
344 osmotrophic feeding (Schultzhaus and Shaw 2015; Richards and Talbot 2018), depend on this
345 process. Cell membrane control and remodelling involve a class of proteins collectively known
346 as the “endosomal sorting complexes required for transport” (ESCRT). The ATPase Vps4 is
347 one of the five distinct multimeric protein complexes of the ESCRT machinery (Williams and
348 Urbé 2007) and has a confirmed role in fungal extracellular vesicle formation (Oliveira et al.
349 2013). Vps4 is recruited to the site of vesicle formation and, through ATP hydrolysis, provides
350 energy and drives the completion of vesicle formation (Baumgärtel et al. 2011; Elia et al. 2011).
351 Recently, the Vps4-binding protein Vfa1 (Vps4-associated-1) was identified in an
352 overexpression study of all putative genes of *Saccharomyces cerevisiae* aiming at exploring
353 vacuole morphology defects (Arlt et al. 2011). Vfa1 overexpression caused altered vacuole size
354 and, later on, it was proved that it is a regulator of the Vps4 ATPase activity (Vild and Xu
355 2014). Our results showed that the Vfa1/Vfa1-like protein (OG0007007) is unique to the
356 aphelids+fungi clade. Thus, this protein arguably constitutes an important molecular
357 synapomorphy that might have played a role in the evolution of the unique feeding and growth
358 strategies found in this clade. Elucidating the unknown functions of the other exclusive genes
359 ancestral to the clade, either derived from HGT (OG0000718, Supplementary Fig. S2,
360 Supplementary Material available on Dryad) or evolved *de novo* (OG0007603, OG0007040,
361 OG0009707, OG0011744; Supplementary Figs. S14, S17, S19, and S20, Supplementary

362 Material available on Dryad), should help unravelling unique common cellular activities in the
363 aphelids+fungi clade.

364

365 *Aphelids and the Early Evolution of Holomycota*

366 Our results strongly support a new phylogenetic framework of Holomycota composed of
367 three main clades: Nucleariida, Rozellida+Microsporidia, and Aphelida+Fungi (Fig. 1).
368 Because they are the closest relatives of Fungi, characterizing aphelid genomes and phenotypes
369 is important to decipher the early evolution of Fungi and, more globally, Holomycota.
370 Although the parasitic rozellids have a more simplified metabolism and, similarly to their
371 microsporidian relatives, have acquired transporters to import nucleotides directly from their
372 hosts (James et al. 2013; Quandt et al. 2017; Dean et al. 2018), they share with aphelids many
373 important structural and biological similarities. Notably, both have free-living flagellated
374 zoospores and feed as endobiotic phagotrophs (trophonts), i.e. preying on the host cytoplasm
375 within the cell wall-defined space. Both aphelids and rozellids penetrate the cell walls of their
376 diverse eukaryotic hosts starting from an extracellular chitinous cyst stage that adheres to the
377 host cell surface. The cytoplasm of this cyst stage then enters the host cell wall-defined space
378 by locally digesting the cell wall and/or exploiting natural spaces between cell wall halves, like
379 in *Tribonema* and diatoms (Karpov et al. 2014; Torruella et al. 2018). Therefore, these
380 characters were most likely present in the last common ancestor of all known Holomycota
381 excluding nucleariids, i.e. the Rozellida+Microsporidia+Aphelida+Fungi group (Fig. 3 and
382 Supplementary Figs. S21 and S22, Supplementary Material available on Dryad). By contrast,
383 nucleariids, the first branch to diverge within Holomycota, typically feed on bacteria and do
384 not exhibit complex life cycles with infective cysts, zoospores, trophonts, and exo- and
385 endobiotic stages (Galindo et al. 2019). Interestingly, the other two holomycotan clades also
386 show a clear feeding preference trend. On the one hand, even if some species parasitize other
387 protists (e.g. amoebae or gregarines), rozellids and microsporidia mostly feed on other
388 opisthokonts (fungi and animals) (Wadi and Reinke 2020). On the other hand, although many
389 fungi have become parasites of animals and many other eukaryotes, aphelids and fungi were
390 ancestrally more specialized in infecting and/or degrading photosynthetic eukaryotes (Naranjo-
391 Ortiz and Gabaldón 2019). Indeed, a wealth of evidence points towards the co-evolution of
392 fungi with green algae and plants as they transitioned to terrestrial environments (Lutzoni et al.
393 2018; Naranjo-Ortiz and Gabaldón 2019; Berbee et al. 2020). Accordingly, we propose to name
394 the supergroup composed of Microsporidia and Rozellida ‘Opisthophagea’ and the supergroup
395 composed of Aphelida and Fungi ‘Phytophagea’ (Fig. 3).

396 These different feeding affinities may have been a major driving force in the evolution and
397 metabolic specialization of these two lineages towards the degradation of, respectively, chitin-
398 or cellulose-based polymers (Fig. 3). Whereas the last common ancestor of Holomycota may
399 have been a bacterivore as most contemporary nucleariids, the common ancestor of
400 Opisthophagea and Phytophagea was most likely already adapted to prey endobiotically on
401 other eukaryotes (Fig. 3). However, whether it fed on opisthokonts, algae or both is difficult to
402 determine (Supplementary Figs. S21 and S22, Supplementary Material available on Dryad).
403 Recently, a study using SSU rRNA gene-based fluorescent *in situ* hybridization on natural
404 marine samples identified an organism that fed inside diatoms and that exhibited a similar life
405 cycle to that of aphelids and rozellids (Chambouvet et al. 2019). In SSU rRNA gene
406 phylogenetic trees, its sequence formed a clade with other marine environmental sequences
407 (chytrid-like-clade-1, NCLC1), which branched as sister to rozellids, although with weak
408 support. Should this relationship be confirmed by future multi-marker phylogenies, the
409 hypothesis that the last common ancestor of Opisthophagea and Phytophagea fed on algae
410 would gain support, such that the dependency of rozellids and microsporidia on opisthokonts
411 would be secondary (Fig. 3).

412

413 Although nucleariids have a non-polarized, non-flagellated cell type, there is a clear
414 similarity between their multinucleated cells and the plasmodial intracellular trophic stage of
415 aphelids and rozellids. This, likely ancient, capacity to grow plasmodial cells may have been a
416 key aspect in the evolution of Holomycota. The adaptation of the Opisthophagea and
417 Phytophagea common ancestor to endobiotic phagotrophy was probably also critical for the
418 subsequent evolution of fungi and microsporidia. Inside the confined environment of the prey
419 cell wall, some of the ancestral holomycotan lineages may have easily lost phagotrophy in
420 favour of extracellular degradation of the algal cell contents by secreting digestive enzymes,
421 leading to the evolution of osmotrophy, a quintessential trait of fungi (Richards and Talbot
422 2018; Torruella et al. 2018). The release of extracellular enzymes within the host cell wall
423 space would have prevented their diffusion to the surrounding environment, disfavouring
424 possible cheaters and competitors. In fungi, the loss of phagotrophy implied the loss of the
425 trophont stage, such that only a cyst-like stage persisted, i.e. a vegetative form endowed with
426 chitin walls and able to emit rhizoids that penetrated host/food cell walls delivering digestive
427 enzymes. Subsequent evolution enriched the enzymatic digestive battery of ancient fungi,
428 making them capable to degrade a variety of complex cell wall polysaccharides (Chang et al.
429 2015; Lutzoni et al. 2018). Many of these digestive capabilities might have been acquired from

430 bacteria. In fact, bacterial symbionts, known for example in nucleariids, have been suggested
431 to protect from algal toxins (Dirren and Posch 2016; Dirren et al. 2017; Galindo et al. 2019)
432 and might have been donors of digestive enzymes through HGT (Garcia-Vallvé et al. 2000;
433 Hall et al. 2005; Marcet-Houben and Gabaldón 2010; Fitzpatrick 2012).

434 Our results suggest that, while fungi entered an evolutionary pathway marked by their
435 feeding specialization and co-evolution with algae and plants, aphelids seem to have largely
436 retained the ancestral phenotype inferred for their common ancestor with fungi.

437

438

439 SUPPLEMENTARY MATERIAL

440 Data available from the Dryad Digital Repository:
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442

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452

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690 FIGURE LEGENDS

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692 FIGURE 1. Phylogenomic analysis of Holomycota. a) Bayesian inference (BI) phylogenomic
 693 tree based on 175 conserved proteins from Lax et al. (2018). The tree was reconstructed using
 694 25 species and 59,889 amino acid positions with the CAT-GTR model and the LG+F+R6+C60
 695 model using the PMSF approximation for maximum likelihood (ML). Numbers on branches
 696 indicate Bayesian posterior probabilities and ML bootstrap values; bootstrap values <50% are
 697 indicated by ---. Branches with support values higher or equal to 0.99 BI posterior probability
 698 and 99% ML bootstrap are indicated by black dots; b) ML bootstrap support for the monophyly
 699 of Fungi (“Fungi”) and two competing hypotheses for the position of Aphelida
 700 (“Aphelida+Fungi” and “Opisthosporidia” (Aphelida+Rozellida+Microsporidia)) as a function
 701 of the proportion of fast-evolving sites removed from the phylogenomic dataset; c) ASTRAL
 702 coalescent-based species tree estimated with the 175 individual protein ML trees. All
 703 phylogenomic trees can be seen in Supplementary Fig. 1 a-e, Supplementary Material available
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705

706 FIGURE 2. Predicted cellular localization of identified synapomorphic proteins shared by
 707 aphelids and fungi. Diagram of an aphelid+fungi ancestor in which black lines indicate the
 708 localization of each protein orthogroup. Orthogroup numbers labelled with an asterisk (*)
 709 indicate proteins derived from horizontal gene transfer events; orthogroup numbers without
 710 asterisk correspond to protein shared by aphelids and fungi which likely evolved *de novo* in a
 711 common ancestor of these two lineages. For the list of proteins see Table S3, Supplementary
 712 Material available on Dryad.

713

714 FIGURE 3. Evolution of key phenotypic traits across Holomycota. Boxes show the ancestral
 715 traits present in the Phytophagea (fungi+aphelids, green), Opisthophagea
 716 (rozellids+microsporidians, orange) and nucleariid (blue) clades and in the last common
 717 ancestors (LCA) of Holomycota and of both Phytophagea and Opisthophagea (grey). For
 718 details on the ancestral state reconstruction, see Supplementary Figs. S21 and S22,
 719 Supplementary Material available on Dryad.

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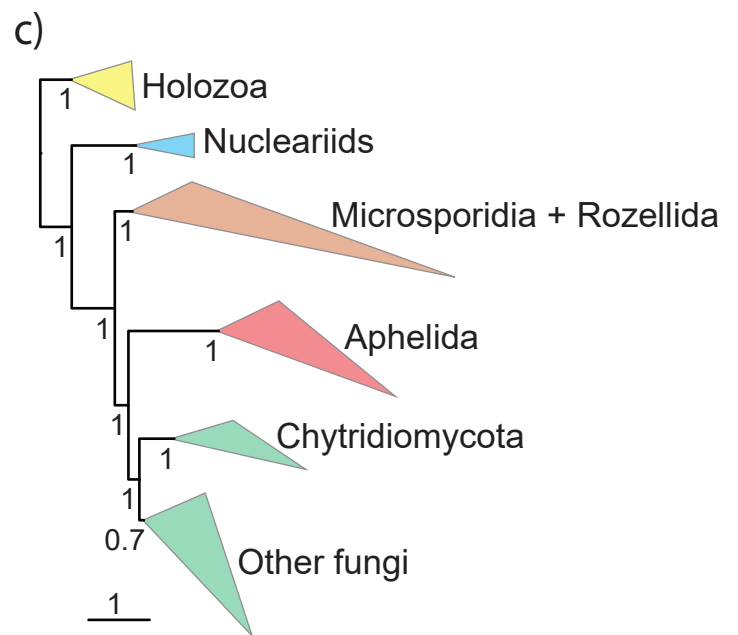
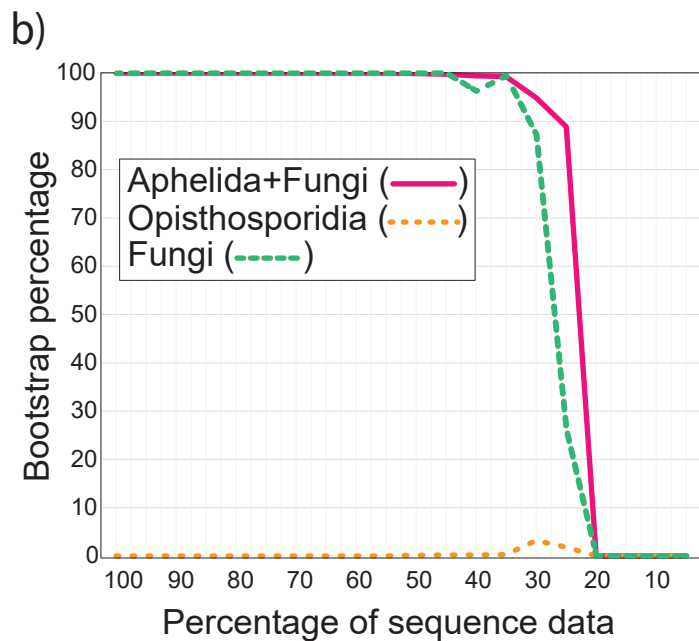
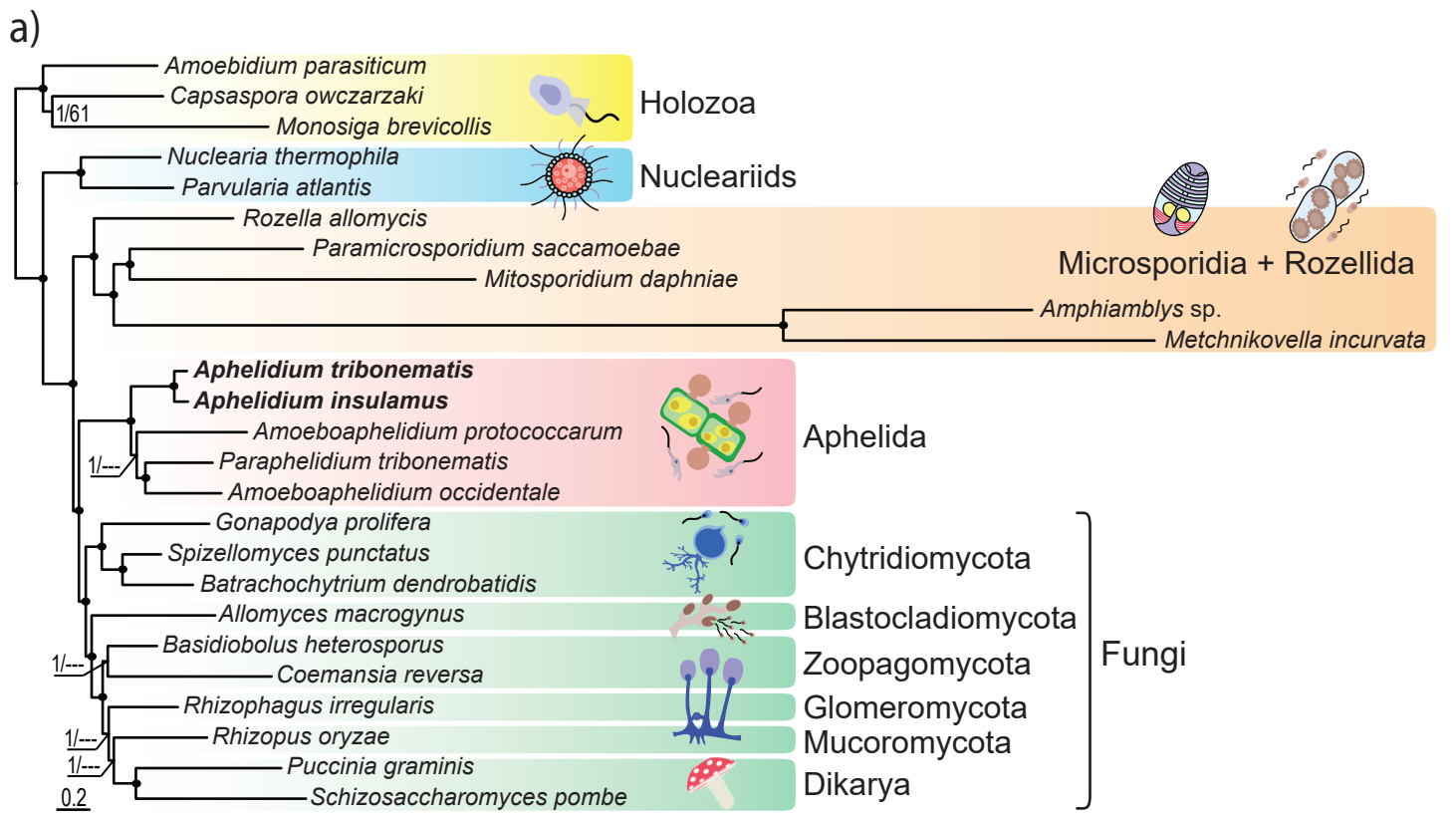


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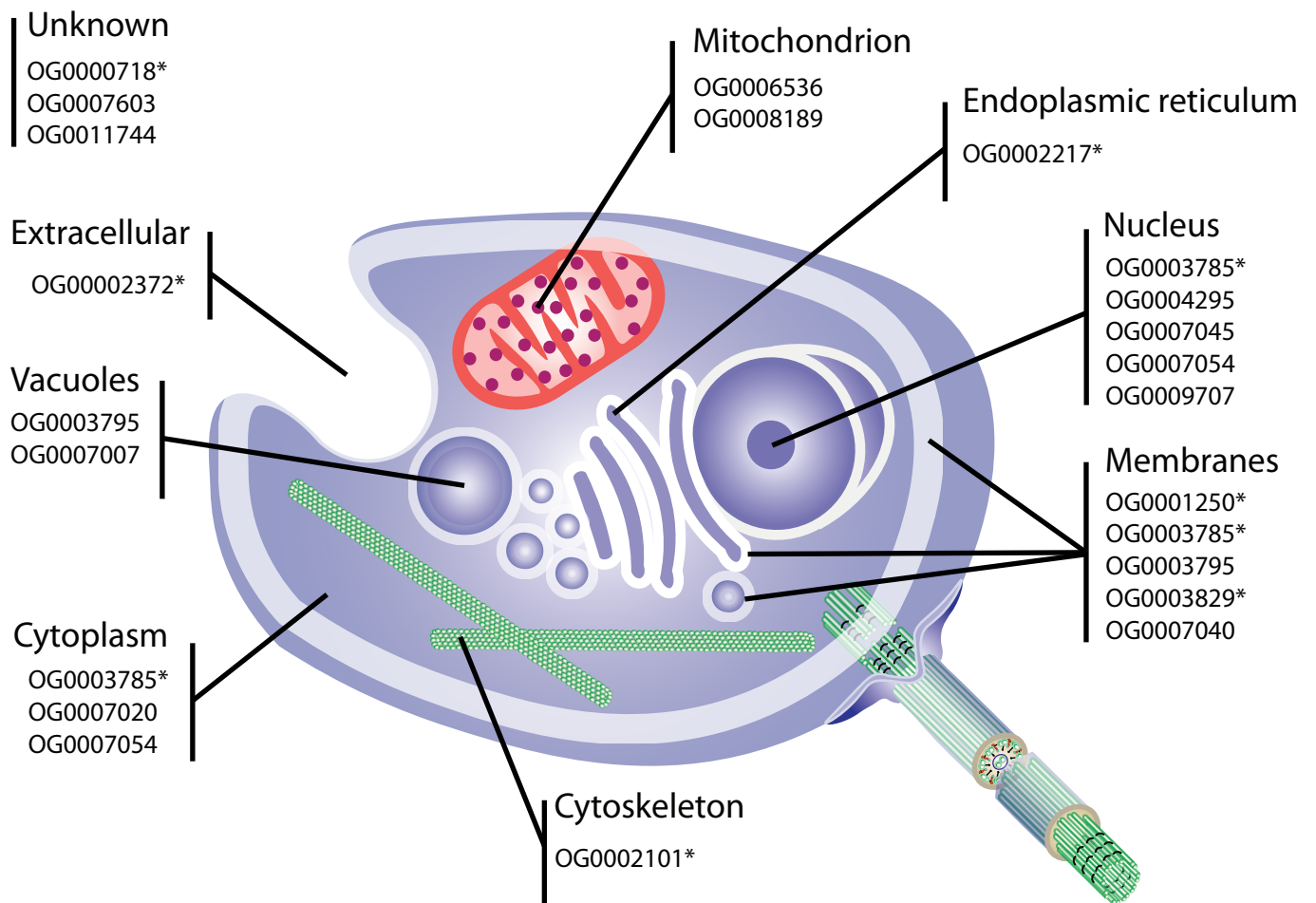


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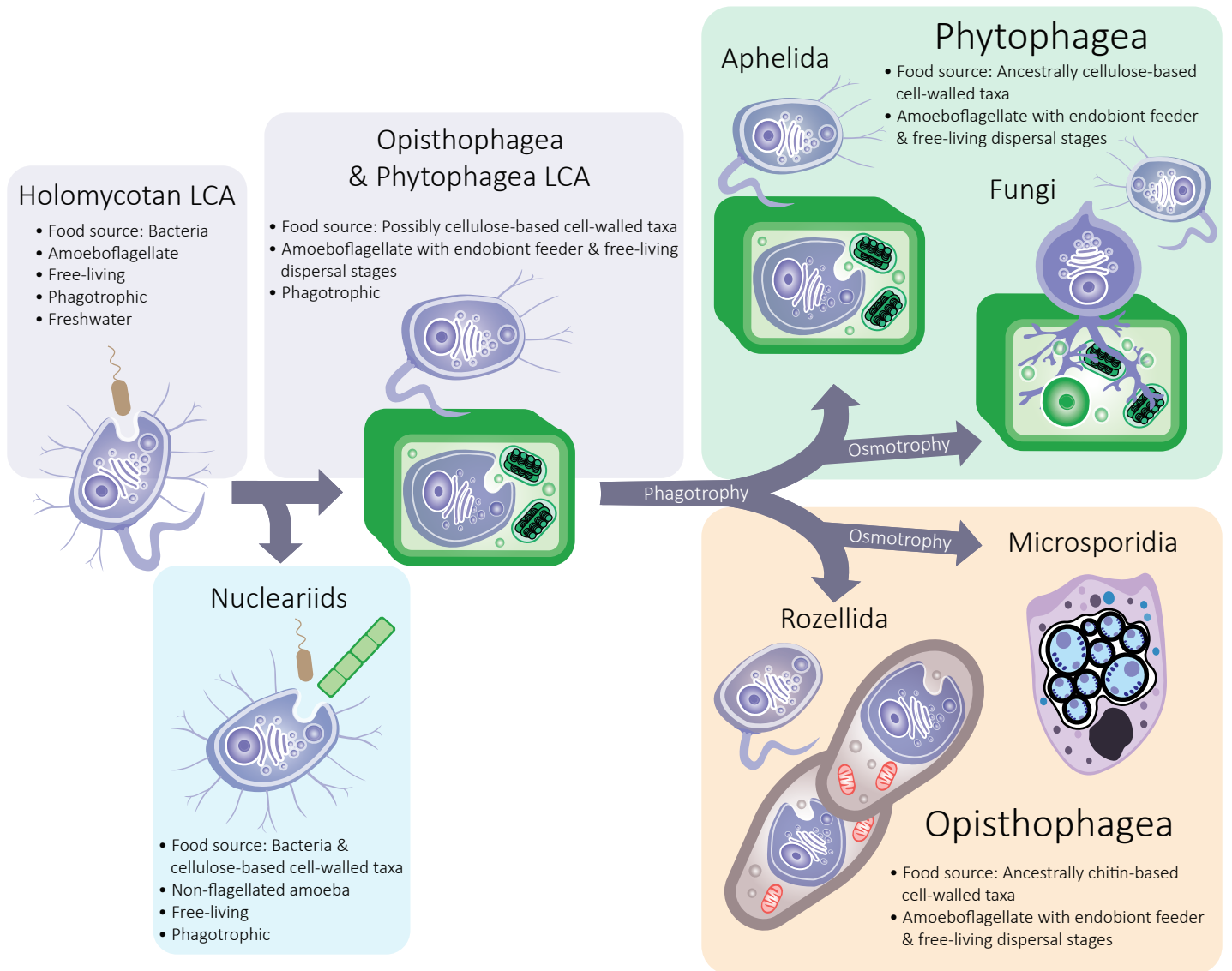


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