

1 Phagocytosis underpins the biotrophic 2 lifestyle of intracellular parasites in the 3 class Phytomyxea (Rhizaria).

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15 Summary

16 Phagocytosis is a complex multi-gene trait of eukaryotes and allegedly one of the very defining features
17 of this group. Although well documented for free-living unicellular eukaryotes and in specific cellular
18 types of animals, data on phagocytosis in intracellular biotrophic parasites are scant. Indeed, the
19 definition of intracellular biotrophy as complete reliance of a parasite on a living host, with which it
20 constantly negotiates for the exchange of nutrients, is at odd with the consumption of particulate
21 matter suggested by phagocytosis. Phytomyxea are intracellular biotrophic parasites infecting a broad
22 group of hosts, ranging from plants to stramenopiles. They belong to the clade Rhizaria, where
23 phagotrophy (i.e., phagocytosis as main mode to acquire nutrients) is the main mode of nutrition. The
24 exact mode of nutrition of the biotrophic phytomyxea, including the agriculturally impactful
25 phytomyxid *Plasmodiophora brassicae*, is still unresolved; despite investigations and the availability of
26 molecular data. For other Phytomyxea, observations are patchy and molecular data altogether lacking.
27 Here, using available genomic and transcriptomic data for Phytomyxea and the *de novo* sequenced
28 transcriptome of the brown algae parasite *Maullinia ectocarpii*, we investigate the likelihood that the
29 genetic machinery underpinning phagotrophy is conserved within the clade. We further document
30 intracellular phagocytosis in *P. brassicae* and *M. ectocarpii* by transmission electron microscopy and
31 fluorescent in situ hybridization. Our investigations confirm that molecular signatures underpinning
32 phagocytosis exist in Phytomyxea and hint at a smaller subset of genes used for intracellular
33 phagocytosis, which is similar between the two parasites. Microscopic evidence confirms the existence
34 of intracellular phagocytosis, which seems to coexist with the manipulation of host physiology typical
35 of biotrophic interactions. In both phytomyxid parasites investigated intracellular phagocytosis has
36 adapted to the intracellular environment and seemingly targets specific organelles. Our findings shed
37 light on the feeding behaviour of Phytomyxea, providing new molecular data for the class; and suggest
38 a paramount and previously unrecognised role for phagocytosis in biotrophic interactions between
39 host and parasite.

40

41 Introduction

42 Often seen as a conserved and nearly universal trait in all major eukaryote lineages, phagocytosis
43 underpins defining eukaryotic features such as the origin of endosymbiotic organelles and of the
44 endomembrane system (Raven *et al.*, 2009; Yutin *et al.*, 2009). Phagocytosis is defined as the
45 interiorization and internal digestion of particles larger than 0,5 μm (Flanagan *et al.*, 2012) and it is
46 assumed to be one of the principal mode of nutrition in the majority of free-living heterotrophic
47 microbial eukaryotes (thereby called phagotrophy), with the remaining feeding by osmotrophy (i.e.
48 extracellularly digestion and/or absorption of molecules via the cell membrane).

49 Despite its pervasiveness among eukaryotes, we owe most of the information on this process to a
50 special group of “professional phagocytes” from the immune system of vertebrate model organisms
51 (Uribe-Querol & Rosales, 2020), for which molecular tools and laboratory experiments are possible.
52 The investigation of phagotrophy and other trophic modes can be challenging in microbial eukaryotes
53 and it relies heavily on microscopic observations and on the labelling/tracking of food items (Keymer
54 *et al.*, 2017; Miura *et al.*, 2017; Godrijan *et al.*, 2022). Even then, the robustness of observational
55 evidence may alone be insufficient in ascertaining the trophic niche occupied by an organism, and
56 sometimes misleading (Not *et al.*, 2007; Moreira & Lopez-Garcia, 2014). Indeed, phagotrophy is often
57 used as an example of a range of “nearly behavioural” traits of microbial eukaryotes, the study of which
58 requires a combination of molecular and laboratory-based investigations (Keeling, 2019).

59 Intracellular eukaryotic parasites can obtain macromolecules from their host via endocytosis, i.e.,
60 phagocytosis of solid food particles and pinocytosis of fluids and the solutes therein. For example,
61 Apicomplexa such as *Plasmodium* spp. (Abu Bakar *et al.*, 2010; Matz *et al.*, 2020) and *Toxoplasma*
62 *gondii* (Dou *et al.*, 2014) ingest and digest macromolecules and pieces of host cell cytoplasm via
63 endocytosis. The kinetoplastid *Trypanosoma cruzii*, has been reported to phagocytotically take up
64 nutrients via the cytostome, a well-defined groove-shaped structure conserved from its free-living
65 ancestors (Chasen *et al.*, 2020). Some intracellular parasites of fungi, oomycetes, and green algae
66 (*Rozella polyphagi* and *R. allomycis*; Fungi, Cryptomycota) have been observed to actively engulf host
67 cytoplasm and organelles, but also to recruit host mitochondria around their thallus, seemingly
68 compensating for their own unstructured and depauperated ones (James *et al.*, 2013; Powell *et al.*,
69 2017). These findings place *Rozella* (together with the above-cited intracellular parasites of animals) in
70 a particular trophic niche where conserved traits from free-living ancestors (e.g., phagotrophy) and
71 derived traits co-evolved with the host (e.g., host manipulation) coexist within the same biotrophic
72 organism.

73 With the notable exception of the photosynthetic chlorarachnids, phagotrophy is assumed to be the
74 main mode of nutrition in almost all the free-living Rhizarians (Cavalier-Smith *et al.*, 2018). Within this
75 clade, Phytomyxea (SAR, Rhizaria) are a class of unicellular eukaryotic parasites living as intracellular
76 obligate biotrophs in plants and stramenopiles in marine, freshwater and terrestrial habitats (Bulman
77 & Neuhauser, 2017; Cavalier-Smith *et al.*, 2018). The class is currently split into three main clades: the
78 orders Plasmodiophorida and Phagomyxida (Hittorf *et al.*, 2020) and the recently described genus
79 *Marinomyxa* (Kolátková *et al.*, 2020). Phylogenetically, Phytomyxea are sister to the free-living
80 Vampyrellida (Sierra *et al.*, 2016; Cavalier-Smith *et al.*, 2018) and Aquavolonida (Bass *et al.*, 2018).
81 Aquavolonida are a group of small, unicellular, free-living phagotrophic flagellates (Bass *et al.*, 2018).
82 Vampyrellida are amoebae with different modes of prey item consumption, ranging from classic
83 phagocytic predation to specialized protoplast feeding, where the prey cell wall is perforated and the
84 amoeba enters the cell and phagocytise it from within (Hess & Suthaus, 2022). Phytomyxea use a very
85 similar strategy to gain access to the host cell, piercing the cell wall with a sophisticated extrusome

86 called Rohr and Stachel (Keskin & Fuchs, 1969; Aist & Williams, 1971). Distinctively, Phytomyxea reach
87 the host cell as flagellated zoospores and penetrate into it as small unicellular protoplasts, later
88 developing into larger intracellular multinucleate feeding plasmodia. Plasmodia can be of two types:
89 short-lived (~ 7 days) sporangial plasmodia, developing into clusters of sporangia (i.e., sporangiosori)
90 and directly releasing infective flagellated zoospores; or sporogenic plasmodia (i.e., sporosori), actively
91 growing as biotrophs inside the living host cell (~ 3-4 weeks) before developing thick-walled
92 overwintering resting spores. During that time sporogenic plasmodia induce hypertrophy of the
93 infected cells which, coupled with induced hyperplasia of the tissue, leads to the formation of galls in
94 the host (Murúa *et al.*, 2017; Olszak *et al.*, 2019). Manipulation of brassicaceae hosts by *P. brassicae*
95 induces hypertrophied infected cells to act as physiological sinks, driving photosynthates from the
96 aerial parts of the plant (Malinowski *et al.*, 2019) and inducing their accumulation as starch grains in
97 the root (Ma *et al.*, 2022).

98 How Phytomyxea feed on their host has never been clearly elucidated and even the trophic mode of
99 the model phytomyxean *P. brassicae* is still debated (Bulman & Neuhauser, 2017). Among the
100 Phagomyxida, the diatom parasites *Phagomyxa* spp. have been observed to ingest the cytoplasm and
101 organelles from their hosts by phagocytosis and accumulate the digested material in pigmented
102 digestive vacuoles (Schnepf, 1994; Schnepf & Bulman, 2000). On the other hand, the lack of a
103 conspicuous digestive vacuole and failure to detect engulfed host organelles has led to conclude that
104 the brown seaweed-infecting phagomyxid *Maullinia ectocarpii* feeds by osmotrophy (Maier *et al.*,
105 2000). Within the Plasmodiophorida, intracellular phagotrophy has been observed in the oomycete-
106 infecting species *Woronina pythii* (Dylewski *et al.*, 1978) and *Octomyxa brevilegniae* (Couch *et al.*,
107 1939; Pendergrass, 1950). Food vacuoles containing residues of cytoplasm and organelles from the
108 host plant *Nasturtium officinale* (watercress) have also been found in *Hillenburgia nasturtii* (formerly
109 *Spongospora subterranea* f. sp. *nasturtii*; Clay & Walsh, 1997; Hittorf *et al.*, 2020). Despite iconographic
110 evidence supporting the existence of phagotrophy in *Plasmodiophora brassicae* (Williams & McNabola,
111 1967; Buczacki, 1983), a clear consensus on whether nutrition is dominated by osmotrophy,
112 phagotrophy or consists of a mix of the two has not yet been reached (Dylewski, 1990).

113 Molecularly, complex and “behavioural” traits such as feeding modes are inherently difficult to
114 investigate, since they are the final phenotypic outcome of a cohort of finely tuned genes involved in
115 a range of overlapping (and often widely conserved) biological processes (Keeling, 2019). *In silico*
116 predictions based on presence or absence of genome-wide molecular signatures identified in
117 organisms known to possess a certain phenotypic trait can be used to infer the likelihood of the
118 existence of that specific trait in other organisms, based on their genomic information (Burns *et al.*,
119 2018). Direct observation, lab-based experiments and analysis of molecular data are complementary
120 and have been successfully used to identify or rule-out phagotrophy in different groups of
121 prasinophytes green algae (Bock *et al.*, 2021; Jimenez *et al.*, 2021).

122 In this study we used genomic and transcriptomic data from the plasmodiophorids *Plasmodiophora*
123 *brassicae* and *Spongospora subterranea* (Schwelm *et al.*, 2015; Rolfe *et al.*, 2016; Ciaghi *et al.*, 2018a,b);
124 and sequenced the transcriptome of the infective stage of the phagomyxid *Maullinia ectocarpii* to
125 detect molecular signatures of phagotrophic behaviour (i.e. protein families present in well-known
126 phagocytes) in the class Phytomyxea. We complemented results from these analyses with fluorescent
127 and electron microscopy observations, to investigate if: (1) intracellular plasmodia engulf organelles
128 and parts of the host cell, (2) the molecular machinery underpinning the phagocytic behaviour is
129 present; (3) intracellular plasmodia express core genes involved in phagocytosis, similarly to other
130 intracellular phagocytes (e.g., *Rozella allomycis*).

131 Material and methods

132 ***M. ectocarpii* transcriptome: biological material, RNA extraction, sequencing and data processing**

133 The model brown alga *Ectocarpus siliculosus* strain Ec32m (CCAP 1310/4) was used as a host for the
134 co-cultivation of *Maullinia ectocarpii* (CCAP 1538/1) for RNA extractions. The pathosystem was
135 maintained in half strength Provasoli medium at 15 °C, with a 12:12 h photoperiod, and an irradiance
136 of 10 $\mu\text{E m}^{-2} \text{s}^{-1}$. Quadruplicates of *E. siliculosus* Ec32m infected with *M. ectocarpii* were generated,
137 harvested after 21 days with a 70 μm cell strainer (VWR, USA), and transferred immediately to ice-cold
138 RNeasy Lysis Buffer (Ambion, Austin, TX, USA), stored overnight at 4 °C, and transferred at -80 °C until used for
139 RNA extraction. Samples in RNeasy Lysis Buffer were thawed on ice, vortexed, and briefly spun down. 500 μL
140 were transferred onto a pre-mixed Bead-matrix (Biozym D1034-MX). Samples were then spun down
141 at 10000 g, 4 °C for 10 min, and RNeasy Lysis Buffer was carefully removed. Samples were immediately snap
142 frozen in liquid nitrogen. Frozen material was subsequently homogenized with a FastPrep (MP
143 Biomedicals, Santa Ana, CA, USA) for 40 s at 6 m s^{-1} . This step was repeated three times and samples
144 were returned into liquid nitrogen in between the three cycles to aid homogenisation and avoid RNA
145 degradation. After the last homogenisation round, samples were transferred into liquid nitrogen and
146 placed on ice. 450 μL buffer RLT (+ β -mercaptoethanol) from the Qiagen RNeasy Plant Mini Kit (Qiagen,
147 Hilden, Germany) were added, samples were vortexed for 30 s and spun down briefly before
148 processing them according to the manufacturer's instructions with an additional ethanol (95%)
149 washing step before RNA elution. RNA quality was tested on an Agilent Bioanalyzer 2100 (Agilent
150 Technologies, Palo Alto, CA, USA). Poly-A selected strand specific library construction and paired-end
151 sequencing (2x 125 bp on a HiSeq 2500 using v4 chemistry; Illumina, San Diego, CA, USA) was
152 performed at the VBCF NGS Unit (Vienna, Austria). Quality of the raw reads was checked using FastQC
153 v0.9.1 (Andrews, 2010). Illumina adapters were removed and only good quality reads (sliding window
154 5 bp; average quality score > 20) with a minimum length of 50 bp were kept using Trimmomatic v0.36
155 (Bolger *et al.*, 2014). Bacterial contamination was removed from the remaining reads using DeconSeq
156 v0.4.3 (Schmieder & Edwards, 2011). Reads from the mock and infected samples were separately
157 mapped against the Ec32m reference genome v2 (Cock *et al.*, 2010) using Bowtie2 v2.2.4 (Langmead
158 & Salzberg, 2012). Unmapped reads from the mock samples were *de novo* assembled into transcripts
159 using Trinity v2.4.0 (Grabherr *et al.*, 2011) with default settings for k-mer size (25 bp) and minimum
160 contig length (200 bp). These transcripts were further used as a reference to filter out host reads from
161 the infected samples and select only reads unambiguously assigned to *M. ectocarpii* (i.e. unmapped
162 reads of this filtering step). Remaining reads were *de novo* assembled into transcripts using Trinity with
163 default settings, thus constituting *M. ectocarpii* transcriptome. Read counts (i.e. gene expression) of
164 the assembled transcripts was estimated using RSEM (Li & Dewey, 2011) included in the Trinity suite.
165 Only transcripts with FPKM (fragments per kilobase per million reads) values greater than one were
166 kept for downstream analysis. Completeness of the transcriptome was verified using BUSCO v5.2.2
167 running in transcriptome mode with the eukaryote_odb10.2019-11-20 reference gene set (Simão *et al.*
168 *et al.*, 2015). *Maullinia* proteome was inferred using the longest open reading frames and the protein
169 coding genes predicted by Transdecoder v5.0.2 (<https://github.com/TransDecoder>) with default
170 settings and used in downstream analyses. Functional annotation of the predicted genes was achieved
171 using InterProScan v5 (Jones *et al.*, 2014).

172 **Additional molecular data**

173 Transcriptome data from an Austrian population of *Plasmodiophora brassicae* were taken from Ciaghi
174 *et al.*, 2018b. Publicly available genomic data were taken from *P. brassicae* strains e3 (Schwelm *et al.*,
175 2015) and PT3 (Rolfe *et al.*, 2016); and *Spongospora subterranea* strain K13 (Ciaghi *et al.*, 2018a).

176 ***In silico* predictions of trophic mode**

177 BUSCO v5.2.2 was run in proteome mode against the eukaryote_odb10.2019-11-20 reference gene set
178 (Simão *et al.*, 2015) to assess completeness of all inferred proteomes, allowing for accurate predictions
179 of trophic modes (Liu *et al.*, 2021). Genomic and transcriptomic data from all three species of
180 phytomyxean parasites in this study show a high degree of BUSCO completeness (< 105 missing
181 BUSCOs over the total 255 BUSCOs in the eukaryota_odb10 database), indicating that their trophic
182 mode can be accurately assigned by TrophicModePredictionTool (Supplementary Figure 1).

183 The TrophicModePredictionTool tool (Burns *et al.*, 2018) was used to predict the trophic mode of the
184 investigated organisms *in silico*, based on the molecular signatures for phagocytic, photosynthetic, and
185 prototrophic capabilities (i.e. organisms capable of synthesising arginine, lysine, threonine, biotin,
186 vitamin B₁, B₂ and B₆). The code (available at <https://github.com/burnsajohn/predictTrophicMode>) was
187 run in the default mode. Prediction scores enumerate the probability that an organism has the genetic
188 toolbox to carry out the indicated function on a scale of 0 to 1. A probability above 0.5 suggests that
189 an organism has the capacity to utilise the indicated function, higher scores indicate a higher degree
190 of confidence.

191 Besides the three main trophic modes listed above, special form of phagocytosis such as that of the
192 extracellular parasite *Entamoeba histolytica* and of the intracellular parasite *Rozella allomycis* are
193 predicted via an emended subset of molecular signatures of phagocytosis. Predictions were visualized
194 as bar charts and by projecting the 4-dimensional probability values onto a 3D tetrahedral shape
195 representing the three trophic modes (or their absence) using scripts modified from the R package
196 “pavo”(Doucet *et al.*, 2013). For static visualization of the trophic mode of an organism, the 3D
197 tetrahedral shape with the summary prediction from each organism plotted onto it is finally rendered
198 as a 2D circular Mollweide projection as described in Bock *et al.*, 2021 and Jimenez *et al.*, 2021. A
199 detailed overview of the genes best matching the predictive molecular signatures are presented in
200 Supplementary Material 1 for the comparison between *P. brassicae* e3 genome and *P. brassicae*
201 transcriptome; as well as for the comparison between *P. brassicae* transcriptome and *M. ectocarpii*
202 transcriptome.

203 **Fluorescent in situ hybridisation and optical microscopy**

204 *P. brassicae* was grown on the host plant *Brassica rapa* var. *pekinensis* (cultivar “Granat”) for 61 days
205 before collection of root galls, thus allowing for the presence of a high number of plasmodia at
206 different stages of development. Plants were grown at 20°C with a 12:12 photoperiod and an average
207 irradiance of 135 $\mu\text{E m}^{-2} \text{s}^{-1}$. Galls were thoroughly rinsed in tap water to remove soil residues and
208 preserved in Histofix 4% (phosphate-buffered formaldehyde solution, Carl Roth, Germany) for ~1 hour.
209 Following fixation, galls were dehydrated in ascending ethanol series: 10 min in 50% ethanol, twice 10
210 min in 70% ethanol and final storage in absolute ethanol. Galls were prepared for FISH staining
211 following the procedure detailed in Schwelm *et al.*, 2016, with few modifications. Briefly, galls were
212 hand cut into thin sections and rinsed for 10 min in hybridization buffer (900 mM NaCl, 20 mM Tris HCl
213 pH 7.5, 35% formamide, 0.01% SDS) before incubation overnight at 46°C in the dark in hybridization
214 buffer, amended with 50 ng of the FISH probe PI_LSU_2313 (Table 1). Samples were washed twice for
215 20 min in washing buffer (900 mM NaCl, 20 mM Tris HCl pH 7.5, 5 mM of NaEDTA pH8, 0.01% SDS) at
216 48°C. Samples were then incubated for 20 min in Hoechst 33342 (Thermo Scientific, USA) diluted 1000X

217 in distilled water, before being mounted in Vectashield (H-1000, Vector Laboratories, USA). *M.*
218 *ectocarpii* was grown on *Ectocarpus siliculosus* Ec32m male gametophyte or *Macrocystis pyrifera*
219 CCAP1323/1 female gametophyte (same culture conditions specified above) for one month before
220 collection. Fixation and FISH staining was achieved in the same way described for *P. brassicae* with the
221 following adjustments. After fixation in 4% Histofix infected algae were incubated for 2 min in 30%
222 H₂O₂ to increase cell wall permeability and then dehydrated in ascending ethanol series. The
223 hybridization was performed at 46°C overnight in the dark in hybridization buffer amended with 50 ng
224 of probe MauJ17 (Table 1). Slides were observed with a Nikon Eclipse Ti2-E microscope equipped with
225 an Andor Zyla 5.5sCMOS monochrome camera and Nikon CFI Plan-Fluor 40x/0.75 NA and 60x/0.85 NA
226 objectives. The excitation wavelength for Hoechst 33342 was 365 nm, whereas it was 490 nm for FISH
227 probes (Table 1). The NIS Elements software (Nikon, Japan) was used for image analysis and post-
228 processing (generation of overlaid images, z-stack analysis and export of z-stack as videos). Final figures
229 were composed using Inkscape 0.92.4 (Inkscape Project).

230

231 **Table 1: Fluorescent In Situ Hybridization probes used in this study**

Probe	Organism/gene	Sequence	Dye	Excitation λ
PI_LSU_2313	<i>P. brassicae</i> /28S rRNA	CCAGGCCTTTCAGCCAAGTA	6-FAM	490 nm
MauJ17	<i>M. ectocarpii</i> /18S rRNA	CACGTCCCTCGTACCCGT	6-FAM	490 nm

232

233 **Transmission electron microscopy**

234 For TEM, *M. ectocarpii* was grown on healthy female gametophytes of *Macrocystis pyrifera* CCAP
235 1323/1 in 1/2 strength Provasoli medium, at 10°C, under 2–6 $\mu\text{E m}^{-2} \text{s}^{-1}$ white light irradiation and 12:12
236 h photoperiod. Biological material was chemically fixed and processed as per Murúa *et al.* (2017).
237 Briefly, the biomass was immersed in a solution composed of 2.5% glutaraldehyde, 0.1 M cacodylate
238 buffer at pH 7.4, 0.5% caffeine, 0.1% CaCl₂ and 0.3% NaCl in Provasoli-enriched seawater (PES) for 2-3
239 days. Post fixation staining was achieved with 1% OsO₄ and 2% uranyl acetate. After dehydration in
240 acetone series, samples were embedded in Spurr resin and polymerised at 60-70°C. Blocks were cut
241 using a Leica UC6 ultramicrotome and counterstained on copper grids with lead citrate. Imaging was
242 achieved with a JEM- 1400 Plus (Jeol, Akishima, Tokyo, Japan) TEM with an AMT UltraVue camera
243 (Woburn, MA, USA). For TEM imaging of *P. brassicae*, root galls of *Brassica rapa* var. *pekinensis* were
244 collected from field material in Weer (Tirol, Austria) in September 2018. Specimens were chemically
245 fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer containing 10% sucrose for 1 h at 4 °C, rinsed
246 with cacodylate buffer and post fixed with 1% osmium tetroxide in 0.05 M cacodylate buffer for 1 h at
247 4 °C. After washing in cacodylate buffer, samples were dehydrated with an increasing acetone series
248 and embedded in EMbed 812 resin. Cross sections of root galls were cut with a diamond knife
249 (Diatome, Switzerland) and an Ultracut UCT (Leica, Austria), mounted on grids, stained with lead citrate
250 and examined with a Libra 120 energy filter transmission electron microscope (Zeiss, Germany). Images
251 were made with a TRS 2 × 2k high speed camera (Tröndle, Germany) and an ImageSP software
252 (Tröndle, Germany).

253 Results

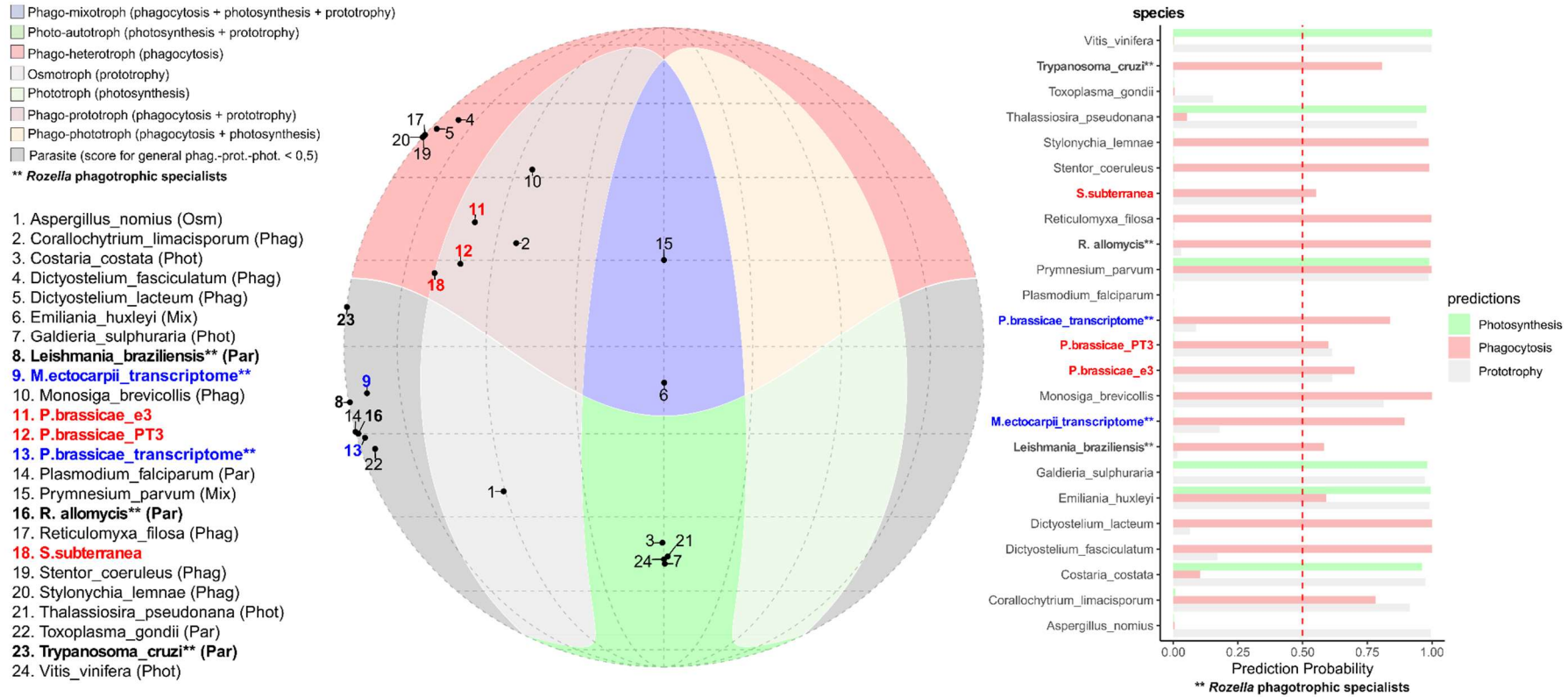
254 *In silico* prediction of trophic modes of Phytomyxea using genomic and transcriptomic signatures

255 All analysed phytomyxids datasets bear molecular signatures of phagotrophy (Fig. 1, Supplementary
256 Table 1). *Plasmodiophora brassicae* (e3 and PT3) and *S. subterranea* (SSUBK13) genomes score high
257 for phago-prototrophy (red numbers 11, 12 and 18 on the Mollweide projection in Fig. 1). The
258 prediction scores from the genome data in *P. brassicae* are approximately 60% for prototrophy (e3 =
259 0.615 and PT3 = 0.612) and are similar for general phagotrophy (e3 = 0.700; PT3 = 0.600). The
260 prototrophy score for *Spongospora subterranea* is lower (SSUBK13 = 0.500, bar chart in Fig. 1), as is
261 the score for general phagotrophy (SSUBK13 = 0.552). When the subset of signatures predicting
262 *Rozella*-like intracellular phagotrophy is considered the probability scores increase to nearly 100% for
263 the genome datasets (e3 = 0.978; PT3 = 0.983 and SSUBK13 = 0.967; bar chart in Fig. 1). The
264 probabilities for photosynthesis and entamoebid-like phagotrophy (a second peculiar mode of
265 phagotrophy mostly observed in extracellular endoparasites such as *Entamoeba*) remain below the
266 threshold of 50% in the genomic data (Supplementary Table 1). When the proteomes inferred from
267 the transcriptomes of *P. brassicae* and *M. ectocarpii* are tested the score for general phagotrophy and
268 prototrophic predictions are very low: in *P. brassicae* the prediction score for general phagotrophy
269 decreases to 0.018 and prototrophy to 0.087, in *M. ectocarpii* phagotrophy scores 0.209 and
270 prototrophy 0.179. The *Rozella*-like phagotrophy remains high with a score of 0.838 in *P. brassicae* and
271 0.894 in *M. ectocarpii* (bar chart in Fig. 1). Presenting these data as a Mollweide projection, the
272 transcriptome datasets are placed with other “Parasite” mapping close to the intracellular fungal
273 parasite *Rozella allomycis* (red numbers 9 and 13 and black number 16 in the Mollweide projection in
274 Fig. 1), while the genomic datasets are in the phago-prototroph area. The assignment to the “Parasite”
275 area in the Mollweide projection highlights a low score (<0,5) for the main trophic categories (i.e.
276 general phagotrophy, prototrophy and photosynthesis), but does not exclude the assignment to
277 specialised sub-categories of phagocytosis (i.e. *Entamoeba* or *Rozella*-like phagocytosis) as highlighted
278 by the bar chart in Fig.1.

279 In *P. brassicae* (for which both genomic and transcriptomic data are available), a detailed look at the
280 molecular signatures highlighted that nearly half (14/29) of the phagotrophy-related genes driving the
281 genome apart from the transcriptome were associated with cilia/flagella (as per their GO term
282 annotation, Supplementary Material 1). Within the predictive model, flagella and cilia are describers
283 of the phago-prototrophic niche, which accommodates organisms using these structures to feed (e.g.
284 Choanozoa such as *Monosiga brevicollis*; Fig. 1).

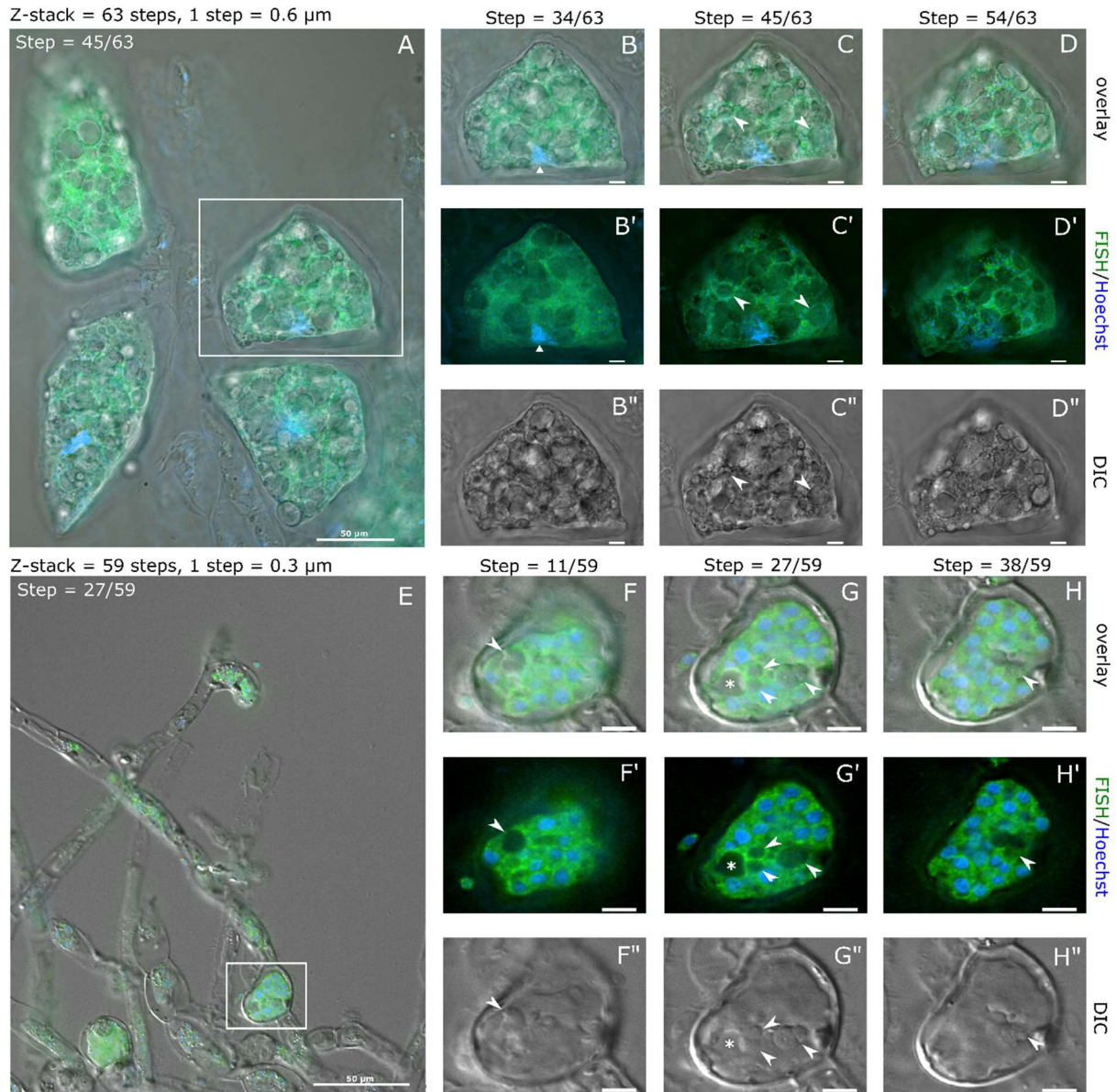
285 On the other hand, trophic predictions for the transcriptomic datasets of *P. brassicae* and *M. ectocarpii*
286 were similar (Fig. 1). In *P. brassicae* genes associated with phagotrophic signatures in the transcriptome
287 were linked to GO terms involving the cytoskeleton (14/40), the cytosol (6/40) and the mTOR
288 complexes (5/40); including the GO terms TORC2 complex, Seh1-associated complex and the lysosome
289 gene RRAGA (Supplementary Material 1). A closer look at the predicted functions highlights their
290 potential involvement in processes such as signal transduction, cell reorganization/polarization,
291 metabolism and cell cycle. In particular Ras GTPases, mTORC1 and mTORC2 complexes are strong
292 descriptors of *Rozella*-like phagotrophic behaviour and describe nearly half (10/21) of the signatures
293 shared between the transcriptomes of *P. brassicae* and *M. ectocarpii* (Supplementary Material 1).

Phagocytosis, prototrophy, and photosynthesis predictions



294

295 **Fig. 1 Mollweide projection showing the position of predicted phagotrophs, prototrophs, and photosynthetic organisms; and bar chart showing the scores of individual**
 296 **prediction probabilities for the same organisms.** Colored regions (red, blue, beige, green) indicate overlapping areas where individual predictions were >0.50. Dark-gray shaded
 297 regions indicate areas where all three predictions were <0.50. Note that phagotrophic specialists (such as *Rozella*-like phagotrophs) do not map the three main trophic categories
 298 and fall in the grey area due to the Mollweide projection only using the general phagocytosis prediction for each organism. Each numbered black dot indicates one of the 19
 299 organisms used as a reference to test the model. The same organisms are represented in the bar chart where prediction probability scores are shown as coloured bars
 300 (green=photosynthetic, grey=prototroph, brown=phagotroph). The 0.5 threshold, above which a prediction is deemed valid, is indicated by the red dashed line. Names in red/bold
 301 indicate the phytomyxid genomes tested in this study whilst blue/bold indicates phytomyxid transcriptomes; names in bold and followed by double asterisks (**)
 302 indicate organisms for which the strongest prediction is *Rozella*-type intracellular phagotrophy.

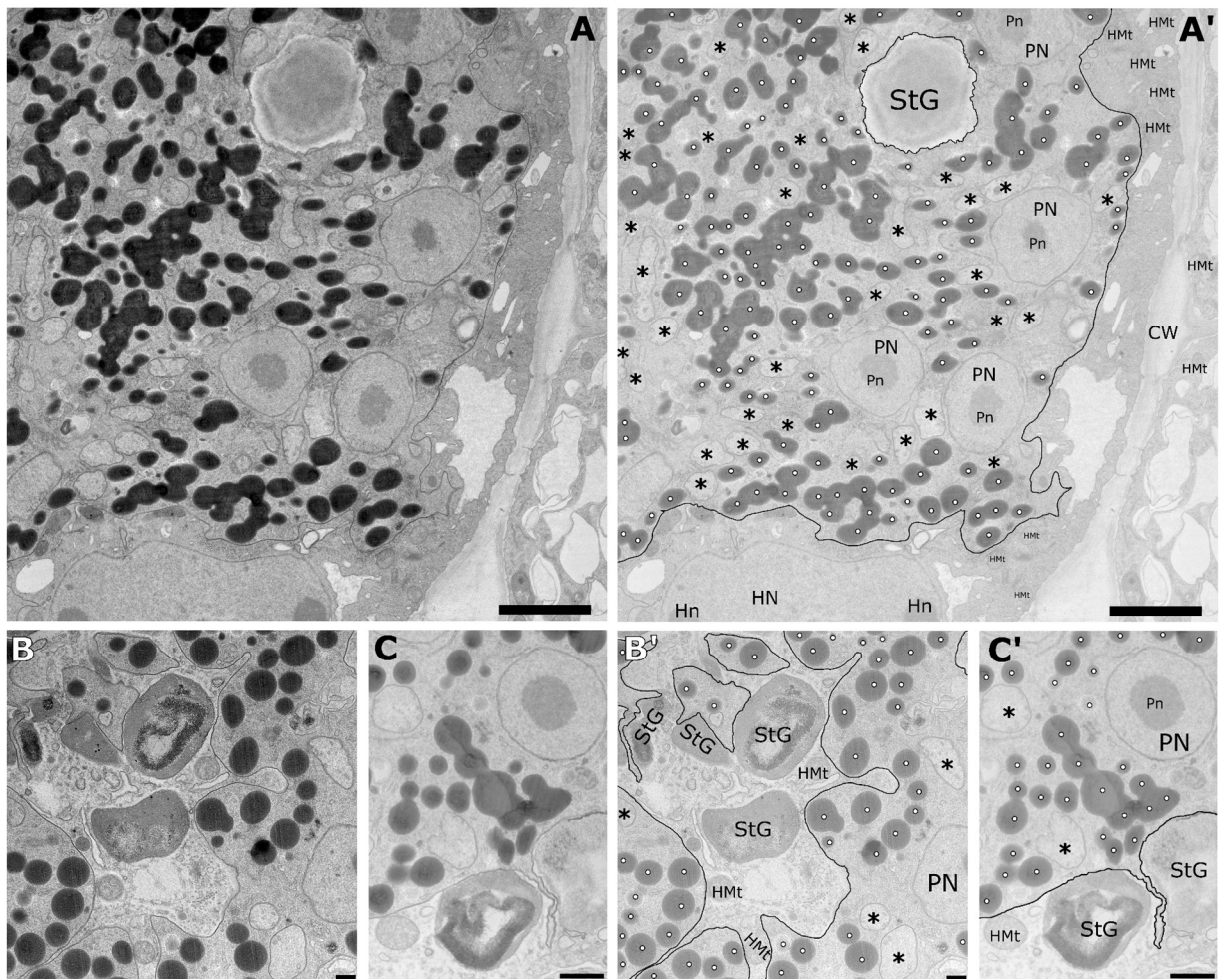


303 **Fig. 2: Optical and fluorescence micrographs provide evidence of the internalisation of host organelles in**
 304 **intracellular plasmodia of *Plasmodiophora brassicae* in *Brassica rapa* var. *pekinensis* (A-D) and of *Maullinia***
 305 ***ectocarpii* in *Ectocarpus siliculosus* Ec32m (E-H).** Images have been captured using differential interference
 306 contrast microscopy (DIC; B''-D'' and F''-H'') and fluorescence microscopy (B'-D' and F'-H') and subsequently
 307 overlaid (A-D and E-H). FISH-probes specific to the 28S rRNA gene of *P. brassicae* and 18S rRNA gene of *M.*
 308 *ectocarpii* were used to highlight the ribosome rich cytoplasm of the parasites (green). Hoechst staining
 309 highlighted the nuclei of both parasite and host (blue) in fluorescence microscopy. White squares in A and E
 310 indicate the plasmodia shown in detail in B-D and F-H respectively. The white triangle in B points toward the
 311 Hoechst-stained host cell nucleus, whilst white arrowheads in C indicate two completely internalised starch
 312 granules. Arrowheads in F-H highlight engulfed algal phaeoplasts, whilst the asterisk in G indicate a vacuole.
 313 Focal planes represent a high (B, B', B'' and F, F', F''), a central (C, C', C'' and G, G', G'') and a low (D, D', D'' and
 314 H, H', H'') layer from z-stacks containing entire plasmodia. In B to D, scale bars 10 μm; in F to H, 5 μm.

315 **Microscopic evidence of phagocytosis in intracellular plasmodia of *P. brassicae* and *M. ectocarpii*.**

316 Microscopic observation of intracellular biotrophic plasmodia of *P. brassicae* and *M. ectocarpii* support
317 phagotrophy of host organelles by the parasites. Mature feeding plasmodia of *P. brassicae* (Fig. 4, A)
318 could be recognized by the high number of small nuclei (Fig. 4, B'-D'; small blue dots) in the absence
319 of cytoplasm cleavage. The plant nucleus was still present and could be distinguished from the parasite
320 nuclei by its larger dimensions (Fig. 4, B-B'; white triangle). Plasmodia filled up the host cells entirely
321 (Fig. 4; A-D; green), leaving little free space within the cell wall. Abundant starch grains were easily
322 identified in DIC microscopy by their shape, size, hyaline texture and tridimensional appearance (Fig.4;
323 B''-D''). A high number of starch grains was located between the plant cell wall and the parasite plasma
324 membrane, pressed against the plasmodium as if superficially "plugged" in membrane pockets (Fig. 4,
325 B-B' and D-D'). Many starch grains were also found to be completely enveloped by the parasite
326 plasmodium, often contiguous to other engulfed and "plugged" starch grains, giving the plasmodium
327 an overall "sponge-like" or "trabecular" aspect in fluorescence microscopy (Fig.4, B'-D'). Two starch
328 grains were entirely surrounded by the plasmodium (Fig.4; C, C' and C'', white arrowheads; videos in
329 Supplementary Material 2) as highlighted by the presence of green hue around them and in the focal
330 planes above and below them.

331 *E. siliculosus* cells infected by mature *M. ectocarpii* were easily distinguishable thanks to the clear signs
332 of hypertrophy (Fig.4 E, white square). The plasmodium shown (Fig. 4, F-H) occupied the majority of
333 the space within the host cell wall, as indicated by the green FISH staining of its cytoplasm. The
334 plasmodium was multinucleated (Fig. 4; F-H and F'-H'; blue signal) and showed vacuolar structures
335 where no green fluorescence could be observed (Fig. 4; F-H and F'-H'; white arrowheads and asterisk;
336 videos in Supplementary Material 2). Some vacuoles contained refractive structures consistent with
337 the phaeoplasts of *E. siliculosus* (Fig. 4; G, G' and G''; white arrowheads) whilst other did not (Fig. 4; G,
338 G' and G''; white asterisk). Phaeoplasts were also observed to be "plugged" in membrane pockets (Fig.
339 4; F, F', F'' and H, H', H''; white arrowhead), much like starch grains in *P. brassicae*. Scansions of the
340 entire volume of the investigated plasmodia along the z-axis are available as videos (Supplementary
341 Material 2); allowing for a better visualization of the host organelles engulfed by the parasites. To
342 further strengthen our observations, we performed a FISH experiment on *M. ectocarpii* infecting the
343 female gametophyte of the kelp *Macrocystis pyrifera*. Even in this case, phagocytosis was observed
344 as highlighted by the observation of phagocytic vacuoles and the late phagocytosis of the host nucleus
345 (Supplementary Figure 2 and Supplementary Material 3).



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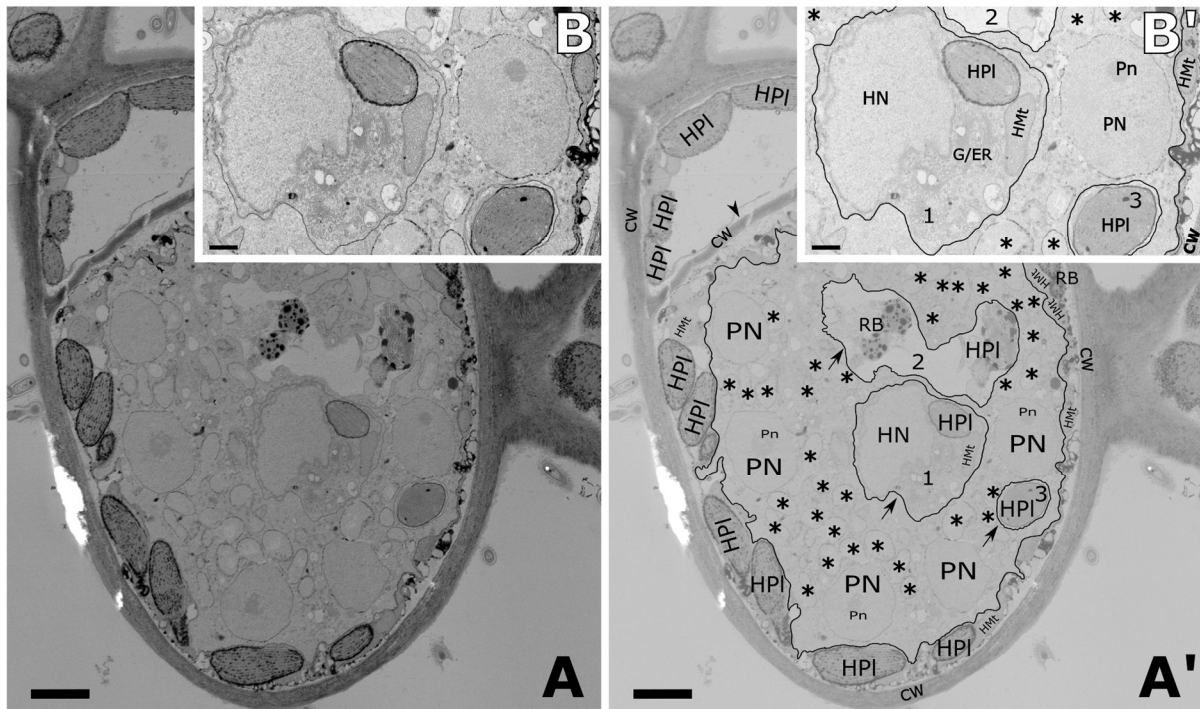
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Fig. 2: Plasmodia of *Plasmodiophora brassicae* growing within the cortical cells of the root of *Brassica rapa* subs. *pekinensis*. Annotations are provided in a separate image from the original picture (e.g. annotations for A in A', etc.) **A-A'**: overview of the interface between the plasmodium and its host. Note the starch granule surrounded by the plasmodium. **B-B'**: Starch grains from desegregated amyloplasts are surrounded by plasmodial protrusions, some of which in closely appressed to the granule surfaces. **C-C'**: Detail of a plasmodial pseudopodium-like process protruding between two starch grains. **In all pictures** note the presence/absence of lipid droplets and the different electron opacity/organisation of the mitochondria, used as main distinctive features to tell apart host and parasite. List of abbreviations: **HN** = host nucleus, **Hn** = host nucleolus, **PN** = parasite nucleus, **Pn** = parasite nucleolus, **white dots** = parasite lipid droplets, **HMT** = host mitochondria, **asterisks (*)** = parasite mitochondria, **CW** = host cell wall, **StG** = starch grains, **black lines** indicate the plasma membrane of the plasmodium. Scale bars: A-A' = 2500 nm, B-B' = 500 nm, C-C' = 1000 nm.



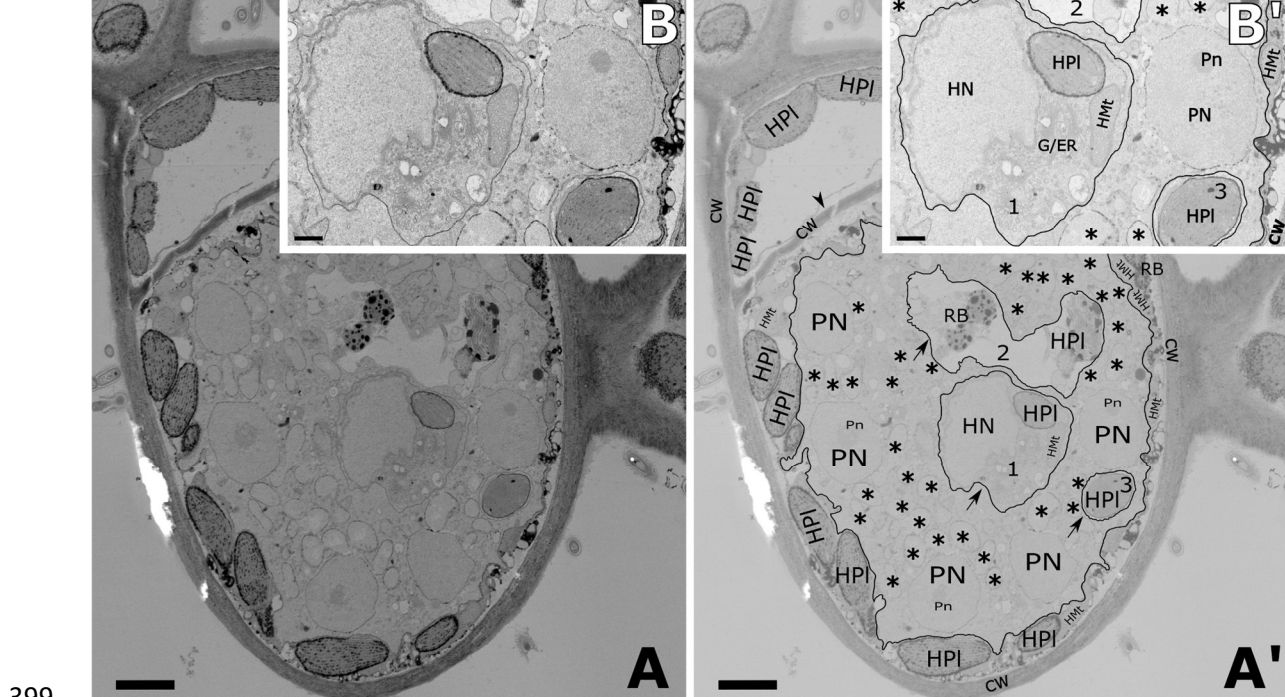
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359 **Fig.3: Plasmodium of *Maullinia ectocarpii* growing within a cell of the gametophyte of *Macrocystis pyrifera*.**
360 Annotations are provided in a separate image from the original picture (e.g. annotations for A in A', etc.) **A-A'**:
361 overview of the interface between the plasmodium and its host. Note the three vacuoles containing algal
362 mitochondria, phaeoplasts and nucleus bound by membrane and surrounded by the parasite plasmodium
363 (arrows). **B-B'**: Close up of A-A' highlighting details of the algal organelles surrounded by the parasitic
364 plasmodium. Note the difference in electron opacity/granularity between the parasite and host cytoplasm used
365 as main distinctive features to tell apart host and parasite. Secondly, note the absence of clear tubular cristae
366 in the putative mitochondria of *M. ectocarpii*. List of abbreviations: **HN** = host nucleus, **Hn** = host nucleolus, **PN**
367 = parasite nucleus, **Pn** = parasite nucleolus, **HPI**= host phaeoplasts, **HMT** = host mitochondria, **asterisks (*)** =
368 potential parasite mitochondria, **CW** = host cell wall, **RB** = residual bodies, **black lines** indicate the plasma
369 membrane of the plasmodium, **Arrows & Numbers** = potential vacuoles, **Arrowhead** = cell wall separating
370 infected and non-infected sectors within the same cell. Scale bars: A-A' = 2500 nm, B-B' = 500 nm, C-C' = 1000
371 nm.

372 **Ultrastructural evidence of phagocytosis in intracellular plasmodia of *P. brassicae* and *M. ectocarpii***

373 Plasmodia and thick-walled resting spores of *P. brassicae* were observed inside the cortical cells of
374 *Brassica rapa* subs. *pekinensis*. Plasmodia can be discriminated from the plant host because of the high
375 amount of lipid globules stored within the cytoplasm (absent from healthy plant cortical cells), the
376 different electron density of the cytoplasm and the electron light mitochondria with sparse tubular
377 cristae (Fig. 2). Multinucleate plasmodia occupy most of the host cell, leaving space only for the host
378 nucleus, small vacuoles and few smaller organelles (like mitochondria) embedded in a film of plant
379 cytoplasm appressed to the cell wall. Parasite nuclei were clearly distinguishable from the plant nuclei,
380 because of their rounder shape and smaller size (Fig. 2, A-A'). The overall shape of the plasmodium
381 was irregularly lobed, to the extent that often it was impossible to clarify whether a single highly lobed
382 or many different plasmodia were inhabiting the same host cell. (Fig. 2, A-A') Lobes of different shape
383 and size were often found surrounding and/or closely appressed to starch grains, originated from
384 desegregated amyloplasts (Fig. 2, B-B'; C-C'). Often plasmodial lobes seemed to encircle and close
385 around starch grains (Fig. 2, C-C') and in one occasion one of those granules was found to be completely
386 surrounded by the plasmodium (Fig. 2, A, StG). The mitochondria of *P. brassicae* were found to be
387 generally electron-translucent and contained less cristae than the lamellar plant mitochondria (Fig. 2,
388 B-B'; C-C'). Mitochondria in thick-walled resting spores were much better defined in their ultrastructure
389 and are overall more electron opaque (Supplementary Figure 4).

390 Feeding plasmodia of *M. ectocarpii* were observed in intercalary and tip cells of the filamentous
391 female gametophytes of *Macrocystis pyrifera* (Fig. 3). Plasmodia readily occupy the whole host cell,
392 initially taking up the space of the central vacuole, thereby pushing the organelles towards the
393 periphery of the cell. *M. ectocarpii* plasmodia are easily discriminated from the host cell by the
394 absence of phaeoplasts and because of the difference in the cytoplasmic electron-density (i.e.,
395 denser in the alga; Fig. 3). Electron dense mitochondria with tubular cristae have been noticed in the
396 algal host. In the plasmodia of *M. ectocarpii* mitochondria are not as visible: putative mitochondria
397 appear as double-membrane bounded electron translucent structures without clearly discernible
398 tubular cristae (



400 **Fig. 3, A-A'; B-B'**). Comparably with observations in *P. brassicae*, in mature *M. ectocarpii*
401 plasmodia/zoosporangia, mitochondria within zoospores show a higher level of structuration, being
402 electron-denser and with well-organised tubular cristae (Supplementary Figure 4). Plasmodia are
403 irregular and sometimes structures similar to pseudopodia can be observed, especially in very young,
404 developing plasmodia which do not yet fill the host cell (Supplementary Figure 3). Vacuoles are often
405 observed within *M. ectocarpii* plasmodia and differ in size and content (Fig. 3 A'-B', arrows and
406 numbers 1-3). Vacuoles can be nearly empty (electron translucent), but most vacuoles are either
407 loosely filled with degraded material (Fig. 3 A'-B', 2) or filled with host organelles and cytoplasm (Fig.
408 3 A'-B', 1 and 3). In Fig.3 B-B' vacuole number 3 can be observed containing a phaeoplast, with little to
409 no space for other structures. A second, bigger vacuole (number 1) contains the host nucleus together
410 with a phaeoplast, one host mitochondrion and host cytoplasm, in turn containing membranous
411 structures interpreted as endoplasmic reticulum and/or Golgi apparatus. An even bigger vacuole
412 (number 2) can be observed in Fig. 3, A-A', within which a clearly degraded phaeoplast and two residual
413 bodies, potentially representing a further stage in phaeoplast degradation, can be observed. The
414 presence of degraded phaeoplasts in vacuole 2 suggests that these have been isolated from the rest
415 of the host cytosol and digested. Presumably, vacuoles 1 and 3 are bound to undergo the same process.
416 The plasmodium itself is multinucleate but it has not yet undergone cytodieresis and zoospore
417 cleavage.

418 Discussion

419 In this study, by analyzing complementary lines of evidence, we demonstrate that phagocytosis is a
420 trait that phytomyxean parasites conserved from free-living Rhizarian ancestors, adapting it to the
421 intracellular environment where it underpins the biotrophic interaction and where it coexists with
422 specialized strategies of host manipulation. Molecular signatures of phagocytosis are present in all
423 phytomyxean datasets analysed; but the model aggregates datasets in different trophic modes
424 according to genome-based and transcriptome-based signatures (Fig. 1). These different predictions
425 can be explained in the light of the polyphasic phytomyxean life-cycle (Liu *et al.*, 2020), where the
426 transcriptome provides a realized molecular snapshot of the feeding stage whilst the genome also
427 contains genetic information on stages other than the intracellular plasmodium (e.g., free-living
428 flagellated zoospores). Genomic signatures identify Phytomyxea as phago-prototrophs whilst
429 transcriptomes of intracellular parasitic stages are best explained by the subset of signatures of the
430 intracellular phagotrophic specialist *Rozella allomycis* (Powell *et al.*, 2017; Fig. 1). Molecular signatures
431 associated with the flagellum are the main drivers assigning *P. brassicae* genomes to the phago-
432 prototrophic niche. Whilst flagella are associated with phagotrophy in certain organisms (e.g.
433 Choanozoa); in Phytomyxea, flagella are exclusively associated with locomotion in the zoosporic phase
434 of the life-cycle but find no implications in nutrition (Barr & Allan, 1982; Parodi *et al.*, 2010; Feng *et al.*,
435 2012). On the other hand, molecular signatures belonging to the Ras GTPases, mTORC1 and mTORC2
436 complexes are shared between *P. brassicae* and *M. ectocarpii* transcriptomes, both assigned to the
437 *Rozella*-like phagotrophic specialists. Ras GTPases are known to control cytoskeletal remodelling and
438 vesicular trafficking in human phagocytes (Wiedemann *et al.*, 2005) and mTORC2 has been linked to
439 cytoskeletal polarization related to budding in yeasts (Loewith *et al.*, 2002). Furthermore, mTOR
440 complexes, particularly mTORC1, are known to be paramount sensors of the nutritional state of the
441 cell acting as a switch between anabolic and catabolic metabolism; and more broadly between growth
442 and proliferation on one hand and autophagy and apoptosis on the other (Sabatini, 2017; Condon &
443 Sabatini, 2019). This hints at a pivotal role of perception of the nutritional state and signal transduction
444 in the intracellular feeding plasmodia of phytomyxids, coupled with cytoskeletal rearrangements that
445 are paramount for phagocytic behaviour.

446 Molecular evidence shows that phytomyxean plasmodia rely on a reduced molecular machinery to
447 perform intracellular phagotrophy, similarly to what happens in the intracellular fungal parasite
448 *Rozella allomycis* (Burns *et al.*, 2018). *Rozella allomycis* belongs to the Cryptomycota, an early-diverged
449 group within the true Fungi (James *et al.*, 2013) which has been demonstrated to be capable of
450 intracellular phagocytosis (Powell *et al.*, 2017). Ultrastructural and molecular data agree in showing
451 that *Rozella allomycis* mitochondria are non-functional and that the parasite relies on the host ones
452 (James *et al.*, 2013; Powell *et al.*, 2017), thus hinting at a complete trophic reliance on the host. Despite
453 the wide phylogenetic distance separating Cryptomycota and Phytomyxea, molecular signatures of
454 phagotrophy from *Rozella* seem to accurately describe the behaviour of intracellular plasmodia in
455 Phytomyxea.

456 Trophic dependency in intracellular plasmodia of Phytomyxea is also supported by ultrastructural
457 observations of *P. brassicae* and *M. ectocarpii*, highlighting big, electron translucent and nearly
458 featureless mitochondria (Supplementary Figure 4). In *R. allomycis* a similar mitochondrial morphology
459 was linked to complete trophic dependency on the host in the intracellular stage (Powell *et al.*, 2017).
460 The smaller and denser mitochondria with defined cristae observed in phytomyxean spores
461 (Supplementary Figure 4; Talley *et al.*, 1978;) and the already reported co-presence of microbodies in
462 zoospores (Tanaka *et al.*, 2001) is consistent with the usage of stored lipids as primary energy source

463 (Held, 1975; Powell *et al.*, 2017). Taken altogether this evidence suggests a metabolic switch from
464 complete reliance on the host during the intracellular growth, to zoosporic reliance on stored fatty
465 acids in Phytomyxea and relates it to distinct changes in mitochondrial activity. Indeed, in contrast to
466 *Rozella*, analyses of the mitochondrial genome of *P. brassicae* did not highlight any particular lack in
467 functional genes (Daval *et al.*, 2018; Stjelja *et al.*, 2019), suggesting that the mitochondrion is still
468 completely functional in other parts of the life cycle.

469 Phagocytosis in Phytomyxea has adapted to their intracellular lifestyle. Feeding plasmodia appear to
470 uptake host structures by protrusion of lobes and invagination of the plasma membrane, again
471 reminding processes observed in *R. allomycis* (Powell *et al.*, 2017), but also akin to the so-called “prey
472 infiltration” strategy used by phylogenetically-related free-living amoebae in the order Vampyrellida
473 (Hess & Suthaus, 2022). Our observations in TEM and fluorescent microscopy show different host
474 organelles engulfed by *M. ectocarpii*, although phagocytosis seem skewed towards phaeoplasts (Fig. 2
475 and Fig. 4 E-H). Likewise, *P. brassicae* seems to target preferentially the host amyloplasts (Fig. 3 and
476 Fig. 4 A-D). Whether this seemingly selective uptake of host organelles is the outcome of real targeting,
477 of chance (phaeoplasts and amyloplasts are the most widespread organelles in the respective host
478 cells) or an observational artefact caused by the delayed digestion of complex plastid-derived
479 organelles remain to be ascertained.

480 However, it is known that host manipulation by *P. brassicae*, beside inducing mitosis and cellular
481 expansion in the host (Olszak *et al.*, 2019); generates a strong physiological sink, driving
482 photosynthates to the infected host cells (Malinowski *et al.*, 2019). Those photosynthates accumulate
483 as starch in amyloplasts, which are significantly more abundant in the infected root cells of brassicas
484 (Ma *et al.*, 2022) where they appear to be superficially “plugged” into the plasmodium surface,
485 reminding of a process of semi-extracellular phagocytosis (named “pomacytosis”, Kamennaya *et al.*,
486 2018). Previous studies highlighted an upregulation of the plastidial MEX1 maltose transporter in
487 infected roots, involved in the export of maltose outside the plastid after starch degradation
488 (Badstöber *et al.*, 2020, Supplementary Figure 5). In this context, we can hypothesise that the
489 pomacytosis-like process observed in *P. brassicae* co-opts phagocytosis to allow for the formation of a
490 close interface between the parasite and the amyloplast without cutting the organelle away from the
491 nucleus, thus allowing for the leaking and fast uptake of soluble sugars such as glucose.

492 Recently identified *P. brassicae* glucose transporters and glucose content in infected roots have been
493 found to significantly increase in late stages of infection (Kong *et al.*, 2022). In the context of a
494 biotrophic interaction that relies on phagotrophy, feeding specifically on amyloplasts has the clear
495 advantage of targeting the host’s carbon storage, whilst at the same time avoiding organelles
496 paramount to the host cell survival and regulation (e.g., the nucleus). This in turn, keeps intact the
497 molecular machinery necessary for the host cell to continue accumulating photosynthetate as
498 amyloplastic starch, giving time to the parasite to complete its life-cycle. Nonetheless, it is unclear
499 whether the targeted phagocytosis/pomacytosis of amyloplasts is an active process or one passively
500 driven by space-constraints within host cells packed with energy rich organelles (Fig. 3 and Fig. 4).
501 Further evidence needs to be produced to confirm this hypothesis; but if confirmed, this would place
502 *P. brassicae* in a particular ecological niche where ancestral phagocytosis provided the baseline to
503 exploit host resources obtained via molecular manipulation co-evolved with specific hosts (Pérez-
504 López *et al.*, 2020; Hossain *et al.*, 2021; Pérez-lópez *et al.*, 2021).

505 Results gathered from *M. ectocarpii* further support intracellular phagocytosis as main mode of
506 nutrition within Phytomyxea. More so since, differently from *P. brassicae* data derived from sporogenic
507 plasmodia, molecular and morphological data for *M. ectocarpii* come from sporangial plasmodia
508 (Maier *et al.*, 2000). Following a conservative and parsimonious interpretation, our results therefore

509 hint at a paramount role of intracellular phagocytosis in both the sporangial and sporogenic phases of
510 the phytomyxean life cycle. *M. ectocarpii* also induces mitosis and cell expansion in its algal host (Maier
511 et al., 2000) but evidence on carbohydrate accumulation in infected tissue has not yet been produced.
512 Brown algae accumulates photosynthates mainly as soluble vacuolar laminaran and cytoplasmic
513 mannitol (Michel *et al.*, 2010; Chabi *et al.*, 2021). It is therefore interesting to notice the early
514 disappearance of the vacuole in cell infected by *M. ectocarpii*. Although consumption of the vacuole
515 seems to be a necessary step of intracellular colonization, simply to provide growth space for the
516 enlarging sporangium; this would also allow for the parasite to immediately consume the major
517 polysaccharide storage within the host cell providing it with rapid energy. However, a first glance at
518 levels of gene expression in *Maullinia*-infected *E. siliculosus* Ec32m did not highlight a clear pattern of
519 upregulation of laminarin / mannitol catabolism or extra-vacuolar transport of carbohydrates
520 (Supplementary Table 2), thus whether *M. ectocarpii* manipulates its host cell carbohydrate
521 metabolism remains unclear. It is worth reminding that the transcriptome analysed here originated
522 from an asynchronous parasite population, where the signal of a possibly transient and punctual
523 interaction between the parasite and the host vacuole could have been diluted in the bulk approach
524 used.

525 In the case of *M. ectocarpii*, phaeoplasts seemed to be a preferred target of phagocytosis. In fact, TEM
526 images show that phaeoplasts in infected cells shrink with the progression of the infection (Fig.2 and
527 Supplementary Figure 3). In depth investigation of plastidial dynamics in infected algal cells is beyond
528 the scope of this study, but the possibility that plastids are directly manipulated before being targeted
529 for consumption by the parasite is possible. If proven, this would hint at a conservation or convergence
530 of the target host organelle within the Phytomyxida. Similar patterns of plastidial shrinkage have been
531 highlighted in the interaction between the intracellular oomycete parasite *Anisolpidium ectocarpii*
532 (infecting *M. pyrifera*), but in this case the decrease in size was interpreted as result of autophagic
533 processes and thus to the reaction of the host against the parasite (Murúa *et al.*, 2020).

534 It is worth bearing in mind that only scarce information is available on the sporogenic stage of *M.*
535 *ectocarpii* (Parodi *et al.*, 2010; Blake *et al.*, 2017) and on Phagomyxida overall (Schnepf & Bulman,
536 2000; Murúa *et al.*, 2017). If, as it is suspected, a sporogenic phase inducing galls formation in adult
537 kelp sporophytes does exist (Blake *et al.*, 2017), an even higher degree of host manipulation can be
538 expected for *M. ectocarpii*, bringing it even closer to its land-dwelling relative *P. brassicae*.

539 Although not common, intracellular endocytosis has been documented in intracellular parasites
540 spanning the taxa Apicomplexa (Spielmann *et al.*, 2020 and references therein), Cryptomycota
541 (Torruella *et al.*, 2018) and Euglenozoa (Etheridge, 2022). The reduced dataset of phagotrophy-related
542 proteins from *Rozella allomycis* correctly describes *Trypanosoma cruzii* and *Leishmania braziliensis* as
543 capable of intracellular phagotrophy (Chasen *et al.*, 2020; Halliday *et al.*, 2020); whilst fails to assign
544 *Plasmodium falciparum* and *Toxoplasma gondii* to this category. Indeed, *P. falciparum* and *T. gondii*
545 are known to use a different set of genes to undertake endocytic nutrients uptake (Spielmann *et al.*,
546 2020) and especially to lack important genes involved in small GTPase (RAS superfamily) and TOR
547 signalling pathways (Van Dam *et al.*, 2011), which are paramount for the predictive model (Burns *et*
548 *al.*, 2018). The apparent proximity of the genetic make-up underpinning intracellular phagocytosis in
549 unrelated Phytomyxea, *Rozella* and trypanosomatids is intriguing, since it hints at the possibility that
550 the smallest subset of genes required for phagocytosis is present in these otherwise unrelated
551 parasites. Although speculative, this hypothesis suggests that intracellular parasites maintaining a
552 phagocytic behaviour despite the well-known process of genome reduction (Keeling & Slamovits,
553 2005) might, when not overly specialised toward their host, make a good model to investigate the
554 very core of the phagocytic machinery.

555 The data presented and discussed here place phytomyxean intracellular parasites half-way between
556 the extremes of specialised biotrophic host manipulation and osmotrophy and generalist phagocytic
557 predation. Growing molecular and microscopic evidence suggests that phagocytosis is a backbone
558 feature of Rhizarians upon which “variations on the theme” brought about the diversification of the
559 group (Anderson, 1978; Hirakawa, 2017; Gerbracht *et al.*, 2022; Hess & Suthaus, 2022). In this context,
560 Phytomyxean are not an exception. It is tempting to speculate that the maintenance and adaptation
561 of phagocytic behaviour is one of the reasons behind the success of this impactful and recalcitrant
562 parasites; allowing them to specialise to certain hosts meanwhile maintaining the ability to feed and
563 propagate within a broader set of organisms (Ludwig-Müller *et al.*, 1999; Maier *et al.*, 2000; Qu &
564 Christ, 2006). Further research on this group of intriguing parasites will surely provide more evidence
565 on the degree of host manipulation/phagocytosis within the class, especially if targeted towards non-
566 model organisms for which data are lacking. Comparative investigations and the exploration of
567 biodiversity surrounding parasites and pathogens proves paramount to deeply understand their
568 biology and potentially devise strategies to counter their effects and broadly foresee the evolutionary
569 trajectories of parasitism.

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810 Supporting information

811 **Supplementary figure 1**

812 Caption: Results of BUSCO analysis of the genomes and transcriptomes of all *Phytomyxid* analysed
813 supporting the high completeness.

814

815 **Supplementary figure 2**

816 Caption: Optical and fluorescence micrographs provide evidence of phagocytosis in intracellular
817 plasmodia of *Maullinia ectocarpii* in *Macrocystis pyrifera* (A-G). Images have been captured using
818 differential interference contrast microscopy (DIC; A''-G'') and fluorescence microscopy (A'-G') and
819 subsequently overlaid (A-G). FISH-probes specific to the 18S rRNA gene of *M. ectocarpii* were used to
820 highlight the ribosome rich cytoplasm of the parasites (green). Hoechst staining highlighted the nuclei
821 of both parasite and host (blue) in fluorescence microscopy. The white arrow in B and F points toward
822 the Hoechst-stained host cell nucleus, whilst white arrowheads in A, B, C, and F indicate putative
823 phagocytic vacuoles. Those are further identified by numbers in A, B and C and highlighted by a white
824 border in F. No algal organelles are clearly discernible within the digestive vacuoles in A-D. Besides
825 absence, this might also be due to the overall coarse appearance of the parasitic thallus and/or to the
826 tilted position of the investigated cell. The plasmodium in E-G is at a later stage of development as
827 highlighted by the complete absence of visible algal organelles (especially phaeoplasts) with the
828 exception of the phagocytised nucleus. Focal planes represent a high (A, A', A'' and D, D', D''), a central
829 (B, B', B''; E, E', E'' and F, F', F'') and a low (C, C, C'' and G, G', G'') layer from z-stacks (steps = 0,1 μ m)
830 containing entire plasmodia. All scale bars are 10 μ m.

831

832 **Supplementary Figure 3**

833 Caption: The picture features a young plasmodium of *Maullinia ectocarpii* in its host alga *Macrocystis*
834 *pyrifera*. The young age of the plasmodium can be inferred from the presence of only one nucleus. The
835 plasmodium (highlighted by a black line in A') bears 4 pseudopodia-like structures (Fig. A', Ps 1-4), 2 of
836 which (Fig. A', Ps3 and Ps4) are used to surround and engulf an algal organelle of difficult identification
837 (Fig A', ?). List of abbreviations : **N** = parasite nucleus, **HMt** = host mitochondria, **HPI** = host
838 phaeoplasts, **CV** = central algal vacuole, **asterisks (*)** = potential parasite mitochondria, **black lines**
839 indicate the plasma membrane of the plasmodium, **Ps** = pseudopodia. Scale bars = 500 nm.

840

841 **Supplementary Figure 4**

842 Caption: Comparison of mitochondria between *Maullinia ectocarpii* (left) and *Plasmodiophora*
843 *brassicae* (right); between feeding plasmodia (top) and spores (bottom). Plasmodial mitochondria are
844 bigger, have fewer, less defined cristae and a sparse electron translucent matrix; compared with the
845 electron-dense and well-defined mitochondria encountered in spores. Note: the figure shows
846 flagellated secondary zoospores for *M. ectocarpii*, whilst thick-walled resting spores are shown for *P.*
847 *brassicae*. Scale bars 200 nm.

848

849 **Supplementary Figure 5**

850 Caption: Changes in the host plant *Brassica oleracea* var. *gongylodes* starch metabolism during
851 infection from *Plasmodiophora brassicae* as per Ciaghi et al., 2018. Log 2 fold changes for significantly
852 differentially expressed genes are shown in the heatmap, which includes numerical transcript
853 identifiers and annotation according to MapMan/Mercator. Up-regulated genes are shaded red, whilst
854 down-regulated genes are shaded blue. The yellow/brown figure symbolizes the amyloplast. The

855 maltose transporter MEX1 and its upregulation in both white and brown root galls as compared to
856 symptomless roots is further highlighted by the white box on the amyloplast wall. Naming conventions
857 follow Ciaghi et al., 2018: WG = small white spindle galls, BG = larger brownish spindle galls and SL =
858 symptomless roots. For details about biological material, data and analyses please refer to Ciaghi et
859 al., 2018.

860

861

862

863 **Supplementary Material 1**

864 Sheet 1 - Caption: Comparison between best-hit genes against molecular signatures of general
865 phagotrophy between *P.brassicae* e3 genome and transcriptome.

866 Sheet 2 - Caption: Comparison between best-hit genes against molecular signatures of Rozella-like
867 specialist phagotrophy between *P.brassicae* and *M.ectocarpii* transcriptomes.

868

869 **Supplementary Material 2**

870 Video – from Fig. 2

871 Because of file size issues, videos from Fig.2 have been made available via the following link to the file
872 sharing service of the University of Innsbruck.

873 <https://fileshare.uibk.ac.at/d/c729cb8ceb6446c1a0ed/>

874

875 **Supplementary Material 3**

876 Video – from Supplementary Figure 2

877 Because of file size issues, videos from Supplementary Figure 2 have been made available via the
878 following link to the file sharing service of the University of Innsbruck.

879 <https://fileshare.uibk.ac.at/d/8799738424d0462b8fae/>

880

881 **Supplementary Table 1**

882 Caption: Values from the TrophicModePredictionTool assigning the proteomes of the listed organisms
883 to 5 different trophic modes. Predictions are deemed significantly accurate when above 0.5 and
884 increase in accuracy with higher values.

885

886 **Supplementary Table 2**

887 Caption: Log 2 fold change expression levels in *Maullinia*-infected *E. siliculosus* Ec32m versus non-
888 infected control for enzymes involved in carbohydrate metabolism. The list of genes presented was
889 compiled based on data from Michel *et al.*, 2010; Chabi *et al.*, 2021 and further manually curated.

890