Electronic Supplementary Materials: Appendix A

Supplementary Methods

The evolution of competitive ability for essential resources

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Supplementary methods

Evolution experiment

We obtained a strain of *C. reinhardtii* (CC1690 wild type mt+) from the Chlamydomonas Center (chlamycollection.org). We then grew this strain in semi-continuous liquid batch culture on COMBO freshwater medium [1], without vitamins, silica and animal trace elements, which are unnecessary for growing green algae. We maintained batch cultures for several months before the start of the evolution experiment. We then plated the cultures onto agar. From the agar plates we selected four random colonies, derived from single cells and inoculated them into liquid COMBO freshwater medium (hereafter referred to as Anc 2, Anc 3, Anc 4 and Anc 5). We then inoculated the chemostats (30 mL total volume) with one of the four monoclonal populations or a genetically diverse population of the original CC1690 population. All populations were composed of a single mating type (+), precluding the possiblity of sex during the experiment. Here we use the term 'population' to refer to each of Anc 2, Anc 3, Anc 4, Anc 5, cc1690 ('ancestors') and their descendant populations ('descendants'), which are the populations evolved in one of seven experimental environments. In total there were five ancestral populations, and 32 descendant populations (three were lost to contamination).

We randomly assigned one chemostat of each of the 5 ancestral populations (Anc 2-5 and CC1690) to one of seven treatments: COMBO, (which we call C, containing 1000 uM N and 50 uM P), nitrogen limitation (N, 10 uM N and 50 uM P), phosphorus limitation (P, 1000 uM N, 0.5 uM P), light limitation (L, 5 µmol photons $m^{-2}s^{-1}$of light,1000 uM N and 50 uM P), salt stress (S, 8g/L NaCl, 1000 uM N and 50 uM P), biotically-depleted medium (i.e. medium previously used to grow seven other species of phytoplankton, (B)), and a combination of salt stress and biotically-depleted medium [2] (BS, 8g/L NaCl). Each chemostat received daily sterile media replacement at a dilution rate of 56% per day via an automated peristaltic pump and was continuously mixed and aerated to prevent heterogeneity in resource availability. We maintained chemostats at 20°C and illuminated under 90 µmol photons $m^{-2}s^{-1}$of light (except the light-limitation treatment) on an 18h light: 6h dark cycle.

62 The biotically-depleted medium was used to investigate the influence that a biodiverse
63 community may have on the evolution of a species by simultaneously depleting the
64 availability of multiple dissolved resources. The salt stress treatment consisted of
65 increasing concentrations of NaCl. We used salt-stress as a point of reference from
66 which to compare the influence of a resource limitation. Salt stress is known to induce
67 evolutionary adaptation (i.e. greater salt tolerance) in *C. reinhardtii [3]*, and in this way
68 we could compare adaptation to limiting resources to another type of stress. We
69 maintained the 'C' cultures in full COMBO medium for the duration of the experiment.
70 Resource-limitation and salt-stress increased incrementally each month until a final,
71 highly-stressful concentration was achieved (ESM Figure S3). The experiment ran for
72 285 days (~285 generations), after which the evolved populations were harvested and
73 plated onto agar.
74
75 ***Culture conditions and acclimation***
76 Prior to R^* assays, we transferred populations from agar plates where they had been
77 maintained for long-term storage after the selection experiment under very low light
78 and temperature (12°C) to limit growth to COMBO medium [1]. We then grew them at
79 20°C and 140 μmol light (hereafter "standard conditions") on a 24 hour light cycle for
80 three days until they reached mid exponential phase. Before the start of each R^*
81 experiment, we allowed each population to acclimate to a relatively low and high
82 resource level for three days. The low resource acclimation conditions were set to
83 match the lowest resource level in the R^* experiments so as to minimize transfer of
84 nutrients from the nutrient replete culture media to the experimental populations. The
85 high resource acclimation level was set halfway between the lowest and highest
86 resource level in the R^* experiments. The low resource acclimation levels were for light,
87 nitrogen and phosphorus: 8μmols/m^2/s, 5 uM N and 0.5 uM P. The high resource
88 acclimation levels were for light, nitrogen and phosphorus: 42 μmols/m^2/s, 80 uM N, 8
89 uM P.
90
91 ***Competitive trait assays***
92 We diluted the 'low' and 'high' resource acclimation cultures to low cell concentrations
93 in 50 mL falcon tubes with COMBO media containing N and P at one of ten resource
94 levels. After diluting each population to very low density (measured as 10 chlorophyll-a
95 relative fluorescence units ("RFU")) at each resource level, we transferred the cultures

96 to the inner 60 wells of 96 well plates (n = 4 replicates per population per resource
97 level, 125 uL per well), covered the plates with a Breathe-Easy sealing membrane
98 (Sigma-Aldrich), and moved them to 20°C temperature-controlled incubators
99 (Multitron, Infors HT, Switzerland), which we set to rotate at 100 rpm. We filled outer
100 wells with COMBO to prevent evaporative losses across the plate. We then tracked
101 their growth by measuring chlorophyll-a fluorescence in RFU (excitation=435 nm and
102 emission=685 nm) over time using a Biotek Cytation 5 plate-reader. We used
103 chlorophyll-a fluorescence because it can be used as a proxy for algal biomass [4],
104 particularly during exponential growth from low density. We measured RFUs two or
105 three times a day for three days, long enough to capture the exponential growth phase
106 at all resource levels. For the N^* and P^* experiments, cultures were illuminated at 140
107 μmol photons $m^{-2} \cdot s^{-1}$ of photosynthetically active radiation ('PAR'); for the I^* experiments
108 light levels were as described below.
109

110 For the nitrogen R^* experiment, the N levels were: 5, 10, 20, 40, 60, 80, 100, 400, 600
111 and 1000 um N. For the phosphorus R^* experiment, the P levels were 0.5, 1, 2, 4, 6, 8,
112 10, 20, 35 and 50 uM P. For the light R^* experiment, N and P were 1000 uM N and 50
113 uM P respectively, and light was supplied at one of ten levels: 0.25, 1.5, 5, 12.5, 27.5,
114 50, 82.5, 125, 175 and 250 μmol photons $m^{-2} \cdot s^{-1}$ of PAR. We manipulated light levels in
115 the light experiment using neutral density filters (Solar Graphics™, Clearwater, Florida),
116 which alter the total amount of light supplied without changing light spectrum. We
117 mounted the light filters onto opaque frames, which fit over the plates and prevented
118 unmeasured light from entering the wells from the sides of the plates. We measured
119 experimental light intensities under the filters using a Skye PAR Quantum sensor.
120

121 ***Salt tolerance assays***

122 Similar to the methods for the R^* assays, all ancestral and descendant populations were
123 first transferred from storage on agar plates to liquid batch cultures and grown in
124 standard conditions. They were then transferred to liquid culture to start an acclimation
125 phase in which each of the populations was subjected to one of five levels of NaCl: 0,
126 2, 4, 6, and 8 g L-1 for four days. Each of the populations was then diluted to achieve
127 a final inoculation density of 50 RFU. Populations from each of the acclimation levels
128 were used to inoculate assay cultures with the same level of salt, or 1 g L-1 more (i.e.
129 0 was used to inoculate 0 and 1 g L-1, 2 to inoculate 2 and 3 g L-1, etc.). For the final

130 growth rate assays, each population was grown in 10 mL of medium in 6-well plates,
131 with a single replicate per population x salt level. We estimated salt tolerance by
132 growing populations over a salt gradient of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 g/L for nine
133 days. To estimate growth rates, RFUs were measured once per day for nine days.
134

135 ***Estimating consumption vectors via stoichiometry***

136 We quantified the consumption vectors for nitrogen and phosphorus for each
137 population. The consumption vector [5] quantifies the amount of each of these two
138 resources used by each individual per unit time. We estimated these vectors by
139 measuring the ratio of phosphorus to nitrogen in the biomass of each population as it
140 was growing exponentially [6]. Stoichiometry during the exponential phase primarily
141 reflects the structural pool of nutrients (vs. the storage pool) [7]. We started by
142 inoculating each population into a 400 mL tissue culture flask with COMBO [1]. We
143 then allowed these populations to grow for approximately 1.5 days under standard
144 conditions until they reached their mid-exponential phase. We then harvested the algal
145 biomass by filtering each culture onto one ashed (500 °C) and pre-massed 47 mm
146 Whatman ® glass microfiber filter (grade GF/F) and one 25mm Whatman glass
147 microfiber filter (grade GF/F). We then dried the filters in an oven overnight at 60 °C,
148 and post-massed the 47mm filters to obtain an estimate of total dry biomass per mL of
149 culture filtered. The 47mm filter was used to estimate the elemental carbon and
150 nitrogen content of the biomass on an Elementar vario PYRO cube EA-IRMS, and the
151 25 mm filter was used to estimate phosphorus content using Skalar San++Continuous
152 Flow P/N analyser. The phosphorus samples were first digested and completely
153 oxidized using a peroxydisulfate solution. We diluted the digested samples 1:20 before
154 being run on the P/N analyser.
155

156 ***Estimating cell size***

157 After the final RFU measurements, we fixed the populations in each well by adding a
158 glutaraldehyde fixative solution (0.01% paraformadehyde and 0.1 % gluteraldehyde),
159 and stored the plates at 4°C until later analysis. To estimate cell size, we took
160 Brightfield photos of the base of each well at 10x on a BioTek Cytation 5 imaging plate
161 reader, from which we extracted cell length (using Gen5 software (BioTek version 2.0),
162 which we converted to biovolume, assuming the cells were spheres (i.e. $4/3*pi*radius^3$).
163

164 ***Quantifying genetic changes associated with selection environments***
165 To gain insight into the genetic responses of *C. reinhardtii* to the selection
166 environments, we prepared Illumina HiSeq libraries of the ancestors and descendants.
167 The ancestral populations of all 4 clonal populations and the original cc1690
168 population were plated onto Sueoka's high salt agar [8] and grown on agar plates for 1
169 month. We harvested the lawn of cells by scraping the agar and placing the biomass
170 into microfuge tubes before performing the DNA extraction. The descendant
171 populations were grown in 50 mL liquid batch cultures in COMBO medium in standard
172 conditions for one week. Due to low level bacterial contamination, and to ensure that
173 sequences were highly enriched by *C. reinhardtii*, these cultures were subjected to an
174 antibiotic treatment of 50 mg/L ampicillin and 50 mg/L tetracycline overnight (<24
175 hours), before harvesting the cells for DNA extraction [9]. The cells were then harvested
176 by centrifugation at 4,000 rpm. The DNA extraction protocol was adapted from the
177 Plant Lab protocol (Institute of Life Sciences, Scuola Superiore Sant'Anna, Pisa Italy and
178 [10]).
179
180 DNA sequencing libraries were prepared using the Bioo Scientific NEXTflex Rapid
181 Illumina DNA-Seq Library Prep Kit according to the standard protocol. DNA
182 sequencing was performed on Illumina HiSeq 2500 version 4 using 125 bp paired ends
183 (250 sequencing cycles). After sequencing the read quality was verified using FastQC
184 version 0.11.2. Adaptor and PhiX cleaning were performed using BBDuk version 35.43,
185 using k-mer size 20 for the former. Quality filtering was performed using PRINSEQ
186 version 0.20.4 with a minimum read length of 50 bp, GC range of 15-85% and
187 minimum mean quality score 5. The quality-filtered reads were aligned against the *C.*
188 *reinhardtii* reference version 5.0 [11] using Bowtie2 version 2.2.5. Variants were called
189 from the resulting BAM files using Freebayes version 1.1.0. The resulting VCF files were
190 quality-filtered using bcftools version 1.4 to select above SNP quality 20 and excluding
191 any SNPs closer to 10 bp from any INDEL due to known read mapping errors around
192 such mutations. The filtered VCF files were further processed in R version 3.5.1 using
193 library Tidyverse version 1.2.1 and Bioconductor package VariantAnnotation version
194 1.28.11. The mutations between the ancestors and descendants were determined by
195 comparing their SNP profiles, determined by comparison to the *C. reinhardtii* cc503
196 mt+, reference version 5.0, using custom R scripts. The R code for sequence
197 processing is available at https://github.com/JoeyBernhardt/chlamee-r-

198 star/blob/master/genomics/workflow.R. The DNA sequences have been deposited in
199 the Sequence Read Archive (SRA) under the BioProject ID PRJNA558172.

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