Genetic predisposition towards multicellularity in *Chlamydomonas reinhardtii*

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Abstract

The evolution from unicellular to multicellular organisms facilitates further phenotypic innovations, notably cellular differentiation. Multiple research groups have shown that in the laboratory simple, obligate multicellularity can evolve from a unicellular ancestor under appropriate selection. However, little is known about the extent to which deterministic factors like ancestral genotype and environmental context influence the likelihood of this evolutionary transition. To test whether certain genotypes are predisposed to evolve multicellularity in different environments, we carried out a set of 24 evolution experiments each founded by a population consisting of 10 different strains of the unicellular green alga *Chlamydomonas reinhardtii*, all in equal proportions. Twelve of the initially identical replicate populations were subjected to predation by the protist *Parmecium tetraurelia* while the other 12 were subjected to settling selection by slow centrifugation. Population subsamples were transferred to fresh media on a weekly basis for a total of 40 transfers (~600 generations). Heritable multicellular structures arose in four of 12 predation-selected populations (6 multicellular isolates in total), but never in the settling selection populations. By comparing whole genome sequences of the founder and evolved strains, we discovered that every multicellular isolate arose from one of two founders. Cell cluster size varied not only among evolved strains derived from different ancestors but among strains derived from the same ancestor. These findings show that both deterministic and stochastic factors influence whether initially unicellular populations can evolve simple multicellular structures.

38 **Introduction**

39 A longstanding question in population biology centers around the relative roles that
40 stochastic processes (e.g., mutation, recombination and genetic drift) versus deterministic
41 process (e.g., natural selection) play in generating evolutionary trajectories and outcomes. In
42 many instances of convergent evolution, the deterministic process of natural selection seems to
43 prevail (1-3). For example, on each of the Caribbean's four largest islands (Puerto Rico, Jamaica,
44 Cuba and Hispaniola), lizards of the genus *Anolis* exhibit four basic morphologies: slender grass-
45 dwelling forms with long tails, ground-dwelling forms with long legs, short-legged forms that
46 creep on twigs, and canopy-dwelling forms with large toe pads. These four body types
47 independently adapted to similar habitats on each island, making it one of the best-studied
48 examples of how evolutionary outcomes can be predictable (2). On the other hand, stochastic
49 processes, such as a lineage's ancestral history and the levels of variation maintained by
50 mutation and drift, can make evolutionary outcomes largely unpredictable (4). For instance, in
51 *Escherichia coli* and *Salmonella typhimurium* resistance to the antibiotic streptomycin caused
52 lower rate of protein synthesis, thereby reducing the fitness of cells when the drug was not
53 present (5, 6). This fitness cost can be diminished by compensatory mutations at other sites in the
54 bacteria's genomes (7, 8). However, when many of those compensatory mutations were
55 introduced into wild-type, streptomycin-sensitive backgrounds, they were found to be
56 detrimental, indicating that the fitness effect of those compensatory mutations depended on
57 genetic backgrounds (5, 6, 9). Had those compensatory mutations occurred without the prior
58 streptomycin-resistant mutations, their evolutionary trajectories and fitness outcomes could have
59 been very different.

60 Laboratory microbial evolution has proved to be a useful tool for investigating under what
61 conditions stochastic versus deterministic factors exert the stronger influence on adaptation
62 evolution. Well-controlled, highly replicated experiments can be carried out for hundreds or even
63 thousands of generations under different types and/or intensities of selection. Such studies have
64 repeatedly shown that similar environments tend to select for similar phenotypes, suggesting that
65 evolution can be deterministic and predictable. For example, all 12 lines in Richard Lenski's
66 long-term evolution experiment (LTEE) in *E. coli* evolved faster growth and larger cell size than
67 the ancestor (10), as well as similar overall patterns of gene expression (11). In asexual haploid
68 *Saccharomyces cerevisiae* populations, sterility and whole genome duplication repeatedly
69 evolves (12, 13). Of course, stochastic processes like mutations can produce these predictable
70 results, provided that the genetic architecture of a selectively advantageous phenotype is
71 sufficiently complex that it can be approached via different mutational trajectories (14). But
72 sometimes, stochastic events like mutations can produce wholly unexpected outcomes. For
73 example, in one of the twelve LTEE *E. coli* populations that had evolved for >31,000 generations
74 in glucose-limiting, citrate-buffered medium, a weakly beneficial mutation followed by a
75 refining mutation together conferred the ability to exploit citrate (Cit^+) as a growth substrate.
76 These mutations were strongly selected, resulting in a Cit^+ dominated, Cit^+/Cit^- population (15-
77 17). A diagnostic feature of wild-type *E. coli* is its inability to ferment citrate under oxic
78 conditions. Thus, this two mutation combo, a tandem duplication that captured an aerobically
79 expressed promoter for the expression of a previously silent citrate:succinate antiporter gene,
80 *citT*, and a promoter mutation that activated expression of C_4-dicarboxylate:H^+ symporter *dctA*,
81 brought about a key evolutionary innovation (17). This innovation, which was contingent on an
82 earlier mutation in the citrate synthase gene *gltA*, benefited from even earlier mutations that

83 increased acetate production after the optimization of glucose metabolism, fundamentally altered
84 the ecological and evolutionary trajectory of the affected population (15, 18).
85 While the roles that stochastic versus deterministic factors play in producing
86 microevolutionary outcomes have been assessed in the lab, it is less clear how such factors have
87 impacted macroevolutionary outcomes, including major evolutionary transitions in the history of
88 life (19). Among these transitions is the evolution of multicellular life-forms from unicellular
89 ancestors, a transition that has occurred repeatedly across the Tree of Life (20-23), and that has
90 served as a gateway to further innovations, such as division of labor among cells, including
91 germ-soma differentiation (19). Historically, our understanding of the mechanisms that have
92 driven this transition has derived chiefly from paleontology (24-26), comparative morphology
93 (27-29), and genomics (30-37), all of which are retrospective in nature. However, in recent years
94 a number of investigators have adopted a prospective approach, and shown that simple, heritable,
95 obligate multicellularity can evolve under selection in the laboratory (38-42). Here, using
96 laboratory evolution we investigate the extent to which two different deterministic processes,
97 selection mode and genetic ancestry, enable a unicellular eukaryote, the green alga
98 *Chlamydomonas reinhardtii*, to make the evolutionary transition from single cells to
99 multicellularity.
100 *C. reinhardtii* is a member of the volvocine algae (Division Chlorophyta), a group that
101 encompasses species exhibiting different levels of complexity that range from single-celled *C.*
102 *reinhardtii*, to colonial *Gonium pectorale*, to fully differentiated *Volvox carteri*. Two prior
103 evolution experiments generated heritable multicellularity in *C. reinhardtii* via selective
104 regimens that favor increased size. Using settling selection, Ratcliff, Herron (41) evolved
105 amorphous multicellular clusters of $>10^2$ cells bound to one another by a transparent

106 extracellular matrix. Using predation selection by the protist *Paramecium tetraurelia*, Herron,
107 Borin (42) evolved a variety of stereotypical multicellular structures, consisting of 2^2 - 2^5 cells
108 surrounded by a common cell wall. These structures bear a striking resemblance to morphologies
109 exhibited by some of the more 'primitive' multicellular members of the volvocine algae, e.g.,
110 *Pandorina* and *Yamagishiella*.

111 Unfortunately, instead of starting from the same ancestral strain, the ancestors used in these
112 two selection experiments consisted of different outbred populations, making it impossible to
113 establish whether it was ancestral genotype or the selective regimen that determined which form
114 of multicellularity evolved. To test whether certain genotypes are predisposed to evolve
115 multicellularity in certain forms, or if particular environments select for specific multicellular
116 phenotypes, we carried out two sets of evolution experiments each founded by a population
117 consisting of 10 different strains of *C. reinhardtii*, all in equal proportions. Twelve initially
118 identical replicate populations were subjected to predation by *P. tetraurelia* and 12 replicate
119 populations were subjected to settling selection. Subsamples of these populations were
120 transferred to fresh media every week for a total of 40 transfers (~600 generations). Because the
121 medium used in this experiment contains abundant nitrate, we expect all reproduction to be
122 asexual (the entry into the sexual cycle is controlled by nitrogen deprivation in *C. reinhardtii*,
123 (43)). Founder strains included two lab strains and eight field isolates, each of which was
124 phenotypically (e.g., cell sizes and chlorophyll content) and genetically different from the others
125 (44). In particular, each of our founder strains contained unique stable polymorphisms that serve
126 as markers for identifying the probable ancestor(s) of evolved multicellular strains.

127 In our experiments, after ~600 generations, heritable, obligate multicellular structures arose
128 in four of 12 predation-selected populations, but never in the settling rate selected populations.

129 We found that only two of the original ten strains were 'parents' for these multicellular
130 descendants, suggesting that certain genetic backgrounds may predispose those strains to evolve
131 multicellularity under this selective regimen. However, the size and structure of the multicellular
132 types were different even in isolates originating from the same 'parent' strain, indicating that
133 stochastic processes like mutation accumulation can lead to strikingly different multicellular
134 forms in populations evolved under the same condition. Together these findings show that both
135 deterministic and stochastic factors can play important roles in shaping the path by which
136 populations evolve simple multicellularity.
137

138 **Materials and Methods**

139 *Experimental evolution*

140 A list of the *C. reinhardtii* founder strains used in this study is provided in **Table 1**. Strains
141 were selected on the basis of their geographic isolation from one another, their phenotypic and
142 phylogenetic diversity, and the fact that the genome of each strain has been sequenced (44). In
143 this study, each founder strain was grown to stationary phase (~6-8 x 10^6 cells/mL) in TAP
144 liquid medium (45); equal volumes of those cultures were mixed and used as the master stock to
145 start the selection experiment. Twelve replicate populations were subjected to predation selection
146 by *Paramecium tetraurelia* (Carolina Biological Catalogue #131560) as described by Herron,
147 Borin (42) and 12 replicate populations were subjected to settling rate selection (41) (**Figure 1**).
148 Each population was cultured on a 14:10-hour light:dark cycle at 22.5 °C in 15 mL of COMBO
149 medium. COMBO is a fresh water medium suitable for co-culturing algae and zooplankton (46).
150 We used COMBO for both predation and settling rate selection experiments so that the two
151 treatments could be compared. Subsamples of each population were transferred to fresh COMBO

152 medium every week for a total of 40 transfers, with approximately 15 generations elapsing
153 between transfers. For experiments under predation selection, 1.5 mL of co-cultured *C.*
154 *reinhardtii* and *P. tetraurelia* were transferred to fresh medium every week. For the settling rate
155 selection, each population was transferred to a 15 mL Falcon tube and centrifuged at 100 x *g* for
156 10 s and only the bottom 0.25 mL was transferred to fresh medium. Under both types of
157 selection, the bottleneck size at each transfer was approximately 1-2 x 10^6 cells.

158 Every two months (i.e., every eight transfers or 120 generations), 0.1 mL samples of each
159 experimental population were diluted 100-fold and 30 μL was plated on 0.5% TAP agar to
160 screen for multicellular isolates. Unicellular isolates are normally motile and form large colonies
161 on 0.5% agar plates, whereas multicellular isolates are normally non-motile and form small
162 colonies (preliminary data not shown). Three small colonies per population (presumably
163 multicellular isolates labelled "A", "B", and "C") were picked up using sterile toothpicks,
164 inoculated into 2 mL of TAP medium in 24-well plates, incubated overnight and screened the
165 next day for multicellular phenotypes by visual examination under light microscopy. Isolates that
166 demonstrated multicellular phenotypes were diluted 10-fold, then 30 μL was replated on solid
167 medium. The same procedure was repeated three times sequentially to ensure that each isolate
168 was clonal and that multicellular phenotypes of isolates from the predation treatment were not
169 transiently induced by the presence of the *P. tetraurelia* predator. All founder strains used to start
170 the evolution experiment and evolved multicellular isolates were cryopreserved at -80°C using
171 the GeneArt Cryopreservation Kit for Algae (Invitrogen, Thermal Fisher Scientific).

172 *Cells per cluster in the multicellular isolates*

173 At the end of our evolution experiments we obtained pure culture isolates of several
174 multicellular strains. To understand how cluster size distribution patterns varied among strains
175 over a 6-day incubation period as well as the maximum number of cells per cluster, we subjected
176 each strain to epifluorescence microscopy. Cells of evolved strains and their ancestors were
177 scraped from TAP agar plates (3-4 inoculation loops, 0.09 cm in diameter), inoculated into
178 15 mL of TAP medium then cultured for five days to high density (approximately 6-8 x 10^6
179 cells/mL). Next, we transferred cultures of each strain into 15 mL of TAP medium at a starting
180 density of 10^4 cells/mL, cultured on a 14:10-hour light:dark cycle at 22.5 °C, and sampled every
181 24 hours over the course of 6-days. We used TAP medium here because it is a standard medium
182 commonly used in *Chlamydomonas* research (45); multicellular phenotypes were stable in TAP.
183 Subsets of cell cultures were removed from well-mixed test tubes and transferred into 1.5 mL
184 Eppendorf tubes (from day 1 to day 6, the amounts of cell cultures transferred were 1200, 900,
185 400, 300, 200, 100 µL). Samples were centrifuged at 14,000 × *g* for 1 minute and the supernatant
186 was replaced by a 50% v/v ethanol-water solution to fix cells and render cell membranes
187 permeable to the fluorescent nucleotide stain DAPI (4′,6-diamidino-2-phenylindole). After a 10-
188 minute fixation period, samples were centrifuged again at 14000 × *g* for 1 minute and the
189 supernatant replaced with distilled water containing 1 µg/mL DAPI. Cells stained with DAPI
190 were kept in darkness for one hour at 25°C then moved to a 4°C refrigerator without light to
191 prevent photobleaching until being imaged on an inverted epifluorescence microscope (the
192 Eclipse Ti series, Nikon).
193 Prior to imaging, samples were removed from the refrigerator and gently pipetted. A 10 µL
194 aliquot of each sample was mounted on glass microscope slides, then multiple images were
195 recorded at 20x magnification using bright field and the DAPI filter set (Excitation/Emission:

196 358/461 nm) overlapping on top of each other. Images were taken of at least 20 clusters or cells
197 per sample, providing a robust dataset for computing the distribution of cluster sizes within each
198 population at a given time point. Next, images were manually screened to demarcate cluster
199 boundaries and record cell number within each boundary using Cell Counter in ImageJ
200 (https://imagej.nih.gov/ij/). Cell number was estimated by counting DAPI stained nuclei
201 confined within cluster boundaries (e.g., **Figure 2**). This procedure was repeated four times for
202 each of the evolved strains and twice for the ancestral strains.

203 *Modes of reproduction in multicellular isolates*

204 Multicellularity can arise either by many cells aggregating (47, 48) or by one cell
205 reproducing clonally and its daughter cells remaining intimately associated (49, 50). In order to
206 distinguish between these two forms of multicellularity, ancestral and evolved strains were
207 imaged using time-lapse transmission microscopy over 72 hours of growth. As before, cells were
208 scraped from TAP agar plates (3-4 inoculation loops, 0.09 cm in diameter), inoculated into liquid
209 TAP medium and cultured for five days to high density (approximately 6-8 x 10^6 cells/mL).
210 Then, each culture was vortexed, transferred 1:100 into fresh TAP medium, and allowed to grow
211 for two more days. Exponentially growing cultures were first mixed by vortexing, then diluted
212 1:10 in TAP medium, whereafter 100 µL from each culture was inoculated into the wells of a 96-
213 well tissue culture plate containing100 µL of TAP medium per well. Well positions for each
214 strain were determined by a random number generator. Wells not inoculated with algae were
215 filled instead with 100 µL of TAP cell-free medium. For time-lapse microscopy, the 96-well
216 plate was imaged at 200x magnification using the Nikon Eclipse Ti inverted microscope, where
217 the field of view was positioned on cells near the center and at the bottom of wells. The time-

218 lapse was run for 72 hours, capturing images of each well every 30 minutes. For each strain, five
219 technical replicates were performed on the 96-well plate.

220 *Identifying the ancestors of evolved multicellular isolates*

221 MATING TYPE ASSAY: To ascertain whether certain ancestral strains were more likely to
222 evolve multicellularity than others, we identified alleles that differed among founder strains
223 (such that each strain has a specific combination), and used those alleles as markers to determine
224 the probable ancestor(s) of evolved multicellular strains. We first established the alleles at the
225 mating type locus in evolved isolates, as the mating types of the founder strains had been
226 previously identified. Each evolved isolate was subjected to PCR-amplification using either the
227 *mid* or *fus1* locus (the *mid* gene is unique to mating type minus (mt-) cells, while the *fus1* is
228 unique to mating type plus (mt+) cells). The *mid* locus was amplified using the primer pair
229 MTM1F (CTG CTG GTA CAA AGG TGT GGC ACG) and MTM2R (CAT GCA GTC TCT
230 CTC ACC CAT TCG GC), which were expected to produce a 750bp piece of the *mid* gene; the
231 *fus1* locus was amplified using the primer pair MTP2F (GCT GGC ATT CCT GTA TCC TTG
232 ACG C) and MTP2R (GCG GCG TAA CAT AAA GGA GGG TCG), which were expected to
233 produce a 434bp piece of the *fus1* gene (51). After identifying the mating type of evolved
234 isolates, we next compared how their whole genome sequences differed from the founder strains,
235 giving us deeper insight into which ancestors produced multicellular isolates.

236 WHOLE GENOME SEQUENCING: Genomic DNA from evolved isolates and the ancestral strain
237 CC-1690 (a wild-type strain in our lab) was extracted, using the *Chlamydomonas* DNA
238 extraction protocol recommended by the Joint Genome Institute (https://www.pacb.com/wp-
239 content/uploads/2015/09/Experimental-Protocol-DNA-extraction-of-Chlamydomonas-using-

240 CTAB-JGI.pdf). Genomic DNA libraries were prepared, and samples were sequenced at 2 x
241 151bp length using the Illumina NextSeq platform at Microbial Genome Sequencing Center
242 (MiGS, https://www.migscenter.com/). For the founder strains other than CC-1690, their
243 sequenced reads were downloaded from NCBI. The quality of sequenced reads of evolved strains
244 and founders was examined by FastQC and trimmed using Trimmomatic-0.36 (*options*:
245 HEADCROP:16 LEADING:30 TRAILING:28 SLIDINGWINDOW:4:15 MINLEN:54). Paired
246 reads were mapped to the Joint Genome Institute v5.5 assembly of the *C. reinhardtii* CC503 mt+
247 genome (52) using BWA-MEM default setting (53). Genomic variants (small polymorphisms
248 such as SNPs (single nucleotide polymorphisms), indels (insertions and deletions), MNPs (multi-
249 nucleotide polymorphisms) and complex events (composite insertion and substitution events))
250 were detected using FreeBayes v1.0.2-29 (*options*: -p 1 -m 3 -q 1) (54). Variants with quality
251 scores greater than 20, read depth greater than 5X, and allele frequencies greater than 0.85, were
252 analyzed. According to a previous study (44), each founder strain contains a genome fingerprint
253 that distinguish them from each other (e.g., see Flowers et al. 2015 **Figure 2B**). Our approach
254 was to generate a filtered genetic variant data set that consisted of nonreference variants
255 observed in all strains together, and identify ancestral identities of multicellular isolates using
256 strain-specific variants along with mating types in founder strains. Sequencing data were
257 deposited at the NCBI Sequence Read Archive with project number PRJNA820479 and
258 accession numbers SAMN27009621-4.

259

260 **Results**

261 *Multicellular structures evolved under predation-selection but not under settling selection.*

262 A founder population of *C. reinhardtii* was created by mixing in equal proportion each the 10
263 strains described in **Table 1**; subsamples of this founder population were subjected to 12-fold
264 replicated experimental evolution according to the scheme depicted in **Figure 1**. Hereafter, we
265 will refer to Predation Selection lines as PS01-PS12 and Settling Rate Selection Lines as SRS01-
266 SRS12. Under the predator selective regime, after 16 weekly transfers we observed multicellular
267 structures in two of the 12 replicate populations, PS05 and PS11. However, evolved isolates
268 from those two populations did not maintain their multicellular phenotypes after serial plate
269 transfers in the laboratory; neither did multicellular morphologies persist in these two
270 populations in the following screen after eight weeks. After 32 weekly transfers, multicellular
271 structures appeared in three populations, PS09, PS10 and PS11; one evolved strain was isolated
272 from each population (PS09-32-A, PS10-32-B and PS11-32-B respectively). Other than PS10-
273 32-B from PS10, the multicellular strains that evolved in populations PS09 and PS11 after 32
274 weekly transfers maintained multicellular morphologies after being plated on solid medium three
275 times in succession, as well as in the experiments described below (**Figure 2: Panels A-B)**. After
276 an additional 8 weekly transfers (40 transfers altogether), multicellular structures were detected
277 in two other predation-selected populations, PS02 and PS12; three evolved strains were isolated
278 from PS02 (PS02-40-A, PS02-40-B, PS02-40-C) and one strain was isolated from PS12 (PS12-
279 40-C). Each of the evolved strains isolated from PS02 and PS12 after 40 weekly transfers
280 maintained their multicellular structures after being plated on solid medium three times in
281 succession, as well as in the experiments described below (**Figure 2: Panels C-F)**. Together
282 these observations indicate that the evolved multicellular phenotypes under predation selection
283 are stably heritable. Under the settling rate selective regime, we observed multicellular structures
284 in two of the 12 replicate populations, SRS05 and SRS 07, after 16 weekly transfers, and again in

285 populations SRS03, SRS08 and SRS11 after 32 weekly transfers. However, isolates from those
286 populations did not maintain their multicellular phenotypes after serial plate transfers in the
287 laboratory. Thus, heritably stable multicellular structures were never observed in any of the
288 settling rate selected populations over the course of 40 weekly transfers, or ~600 generations.
289 In the unicellular ancestors, most cells exist as single cells in their respective populations
290 (**Figure 2: Panels G-H**). By contrast, within each multicellular structure of the evolved strains,
291 cell walls are visible surrounding individual two, four or eight cells, and these are held in place
292 by transparent extracellular matrix (**Figure 2: Panels A-F**). Most evolved strains form clusters
293 approximately 20-100 µm in size, except PS09-32-A, which can form clusters whose diameters
294 range up to several hundred micrometers (**Figure 2: Panel A**). Development in the evolved
295 multicellular strains is strictly clonal. Time-lapse microscopy over 72 hours shows that,
296 compared to the ancestors where dividing cells only transiently form clusters before daughter
297 cells are released (e.g., CC-2936, **Supplemental Video 1**), multicellular isolates develop by
298 parental cells dividing into daughter cells within clusters over multiple rounds of cell division
299 (**Supplemental Videos 2-7**). Because cells within each multicellular cluster are genetically
300 identical, clusters likely function as units of selection (55, 56) under predation selection.
301 *Cells per cluster in the multicellular isolates*
302 In order to determine cluster size distributions for evolved strains, we sampled, stained, and
303 imaged growing cultures every 24 hours over a 6-day period, which approximated the serial
304 transfer interval of our evolution experiments. Four biological replicates were performed for
305 each of the evolved strains, and two biological replicates were performed for the ancestral
306 strains. As distributions tended to be right-skewed, data were represented as boxplots that depict
307 clusters' central tendencies. Large outlier clusters were occasionally present in each population.

308 These outliers, defined as data points that exceeded a distance of 1.5 times the interquartile range
309 (IQR) above the 3rd quartile, were excluded from further analyses.
310 We observed that multicellular cluster size varied among evolved strains over the course of
311 6-days. The median cluster size of multicellular strain PS09-32-A was >80 cells on day-1 and
312 then maintained at >100 cells throughout the growth cycle, with a maximum of 156 cells on day-
313 6 (**Figure 3A**). By contrast, median cluster sizes of PS11-32-B, PS02-40-A, PS02-40-B, and
314 PS12-40-C ranged between 2 and 10 cells, with maximum sizes being reached on day-1 (6, 10, 7
315 and 7 cells, respectively) then gradually decreasing thereafter (**Figure 3B, C, D, F**). The median
316 cluster size of strain PS02-40-C remained at 4 cells between day-1 and day-5 before declining to
317 2 cells on day-6 (**Figure 3E**). Median cluster sizes of the ancestral strains (CC-2936 and CC-
318 1690, see the following paragraph for identification details) remained one cell per cluster over
319 growth cycle, with multicellular clusters occurring occasionally during reproduction (**Figure 3G-**
320 **H**).
321 We found that there was a significant difference among evolved strains and ancestral strains
322 in the median cluster sizes (Kruskal-Wallis Test, $H = 242.22$, $df = 7$, $p << 0.01$). To ensure our
323 comparisons were of similar developmental stages, the median for each strain was measured at
324 the time point at which it reached its maximum value, as in Herron, Borin (42). Particularly, the
325 median cluster size of PS09-32-A (156 cells) was significantly larger than those of other
326 multicellular strains (6, 10, 7, 4 and 7 cells from PS11-32-B, PS02-40-A, PS02-40-B, PS02-40-
327 C, PS12-40-C, respectively) and those of their ancestors (1 and 1 cell from CC-2936 and CC-
328 1690) (**Table 2**). There were no significant differences in median cluster sizes among all the
329 multicellular strains other than PS09-32-A (**Table 2**).
330 *Multicellular isolates evolved from the founder strains CC-2936 and CC-1690*

331 PCR analysis of mating types in the evolved multicellular isolates revealed that all were mt+,
332 suggesting they originated from at least one of the mt+ founder strains (**Supplemental Figure**
333 **1**). Therefore, for the following genomic analysis, we only compared evolved isolates with mt+
334 founders to identify which ancestor genotype(s) they were most likely to have originated from.
335 We performed whole genome sequencing of PS09-32-A, PS11-32-B, PS02-40-A in PS02 and
336 PS12-40-C to an aligned sequencing depth of ~15X, 15X, 14X and 18X using paired end (2 x
337 151bp) Illumina sequencing. One of the mt+ founder strains, CC-1690, was re-sequenced for a
338 separate study (not published) to an aligned depth of 44X using the same Illumina sequencing
339 method. We did not sequence PS2-40-B and PS2-40-C in PS02 owing to the fact that they are
340 phenotypically similar to PS02-40-A, and are likely the same genotype as PS2-40-A, or only
341 differ by a few mutations, as they were isolated from the same population. For the remaining
342 four mt+ founder strains, their sequenced reads were downloaded from NCBI using accession
343 numbers SAMN03272879, SAMN03272875, SAMN03272877 and SAMN03272872 for strains
344 CC-2937, CC-2936, CC-2344 and CC2343, respectively (44). Based on the sequence alignments
345 to ~112 Mb of the *C. reinhardtii* CC-503 mt+ reference genome (52), we generated a filtered
346 genetic variant data set that consisted of 4,714,560 nonreference variants in all strains together.
347 Each strain contains a genome fingerprint based on the location of their genetic variants across
348 17 *C. reinhardtii* chromosomes. By visually comparing the genome-wide distributions of those
349 variants and through direct comparisons, we can infer the most likely ancestors of evolved
350 multicellular isolates in the populations PS02, PS09, PS11 and PS12.
351 Among mt+ founder strains, lab strain CC-1690 is diverged from reference strain CC-503 by
352 90,242 variants in our filtered data set, while the field isolates CC-2937, CC-2936, CC-2344 and
353 CC-2343 differ from CC-503, respectively, by 1,651,964, 1,194,250, 2,102,758, 2,187,600

354 variants in 111,098,438 sites across the reference genome (**Table 3**). Among evolved strains,
355 PS09-32-A, PS11-32-B and PS02-40-A contain 1,169,953, 1,110,266 and 1,144,326
356 nonreference variants, respectively, while PS12-40-C only contains 77,708 variants (**Table 3**).
357 When comparing the genome-wide distributions of nonreference variants between mt+ founders
358 and evolved strains, we found that the distributions in PS09-32-A, PS11-32-B and PS02-40-A
359 visually match to that in CC-2936 while the distribution in PS12-40-C visually match to that in
360 CC-1690 (**Figure 4**). When directly comparing the variants between mt+ founders and evolved
361 strains, we found that > 99% of variants identified in the evolved strains PS09-32-A, PS11-32-B
362 and PS02-40-A matched to the founder strain CC-2936, while 100% of the variants identified in
363 PS12-40-C matched to the founder strain CC-1690 (**Table 3**), suggesting that PS09-32-A, PS11-
364 32-B, PS02-40-A evolved from CC-2936 and that PS12-40-C evolved from CC-1690. The < 1%
365 of variants in PS09-32-A, PS11-32-B, PS02-40-A that did not match to CC-2936 is likely due
366 either to different sequencing methods (e.g., CC-2936 was previously sequenced at 2 x 51bp
367 length using the Illumina HiSeq 2000 sequencer (44) while the evolved strains here were
368 sequenced at 2 x 151bp length using the Illumina NextSeq 2000 sequencer), or to mutations that
369 accumulated in these lines since they were sequenced by Flowers, Hazzouri (44).
370 To further illustrate that specific allele combinations in the founder strains CC-2936 and CC-
371 1690 are also present in their respective evolved isolates, **Table 4** lists 25 loci across different
372 chromosomal locations that differ among five mt+ founders. The nucleotides in those 25
373 positions in PS09-32-A, PS11-32-B and PS02-40-A all match to the ones in CC-2936, while the
374 nucleotides in those positions in PS12-40-C all match to CC-1690 (**Table 4**), indicating that CC-
375 2936 is the ancestor of PS09-32-A, PS11-32-B and PS02-40-A and that CC-1690 is the ancestor
376 of PS12-40-C.

377

378 **Discussion**

379 The emergence of multicellular organisms and the emergence of eukaryotes are widely
380 regarded as major evolutionary transitions in the history of life (57). However, unlike
381 eukaryogenesis, which seems to have been a singular event (58, 59), multicellularity has arisen at
382 least 25 times across widely divergent lineages (20). Within the volvocine algae, multicellularity
383 arose in some lineages as recently as the Jurassic and Cretaceous periods (126-49 million years
384 ago) (37, 60-62). Still, even though multicellularity has arisen repeatedly and in diverse lineages,
385 little is known about the relative roles that stochastic versus deterministic factors play in driving
386 this transition. Previous work has shown that in the lab simple, obligate multicellularity can
387 evolve in the unicellular green alga *C. reinhardtii* under either predation or settling selection (41,
388 42). Here, we used experimental evolution to assess the influences that genetic ancestry and
389 selective environment have on whether single cells become stably multicellular. We established
390 a founder population consisting of 10 different strains of *C. reinhardtii*, all in equal proportion.
391 This founder population was used to generate 24 replicate populations: 12 were subjected to
392 predation by the protist *P. tetraurelia*, 12 were subjected to settling selection that favors large
393 cell clusters that settle rapidly. After ~600 generations, heritable, obligate multicellular structures
394 arose in four of 12 predation-selected populations, but never in the settling rate selected
395 populations. Furthermore, we found that multicellular isolates evolved from only two founders
396 CC-1690 and CC-2936, and that the size and structure of some multicellular isolates from the
397 founder CC-2936 differed from each other. Whether the obligate multicellularity we observed
398 was at a stable point or at some point along a continuum of evolving multicellularity, our results

399 indicate that both deterministic and stochastic factors play significant roles on the trajectories by
400 which populations evolve multicellularity.

401 If the evolutionary transition to multicellularity was purely deterministic, we would expect
402 that similar selective environments would result in similar phenotypes regardless of ancestral
403 genotypes. However, the fact that we observed different types of multicellularity evolve under
404 predation selection, even from the same ancestor (CC-2936), suggests that stochastic processes
405 like the accumulation of random mutations can lead to largely different outcomes. That being
406 said, the large clusters formed by PS09-32-A (> 100 cells most of time) can be costly, as the
407 growth of a colony may be limited because interior cells may not have the same access to
408 nutrients or environmental signals (e.g. oxygen or lights) compared to exterior cells (63, 64). If
409 this is true, then it is possible that PS09-32-A might have evolved a smaller cluster size similar to
410 what we observed in other multicellular isolates had we allowed the experiment to run longer.
411 Such a response to selection would demonstrate that evolutionary process is still deterministic at
412 the phenotypic level.

413 We also found that the multicellular isolates evolved in three of 12 predation-selected
414 populations came from founder CC-2936, while the multicellular isolate evolved in the other
415 predation-selected population descended from founder CC-1690. We did not observe
416 multicellular isolates evolved from any other founders. These results suggest that particular
417 suites of genetic variants had accumulated in those strains prior to our evolution experiments,
418 and that these facilitated the transition to multicellularity in our selective regimen. An inverse
419 question to ask is: why are some founders more resistant than others to selection pressures
420 expected to favor evolution of multicellularity? Were the populations quickly purged of certain
421 founders just because they grew less quickly or to a lower stationary phase density? In other

422 words, were the results biased at the onset because certain strains have poor competitive ability,
423 relative to others? To test these ideas, future work should be directed toward competition
424 experiments among founders, and/or similar evolutions using the same founders where
425 populations are sequenced at different time points to monitor changes in the frequency of allelic
426 markers by which founders can be differentiated.

427 Multiple reasons could explain why multicellularity did not evolve under our settling
428 selection regimen. First, in our experiments settling rate selection was episodic, occurring only
429 once a week, whereas predation selection was continuous. Second, our settling rate selection
430 (centrifugation at 100 *g* for 10 s, with pelleted cells transferred to fresh medium once a week)
431 differed slightly from that of Ratcliff, Herron (41) (100 *g* for 5 s, with pelleted cells transferred
432 every 3 days). Third, so that the two treatments could be compared it was essential that the
433 settling selection and the predation experiments be carried out in same medium. We elected to
434 use COMBO, a defined freshwater medium for co-culturing algae and zooplankton (46) that had
435 been used successfully in our earlier predation experiment (42). Significantly, COMBO does not
436 contain an organic carbon source, therefore, it only supports photoautotrophic growth of *C.*
437 *reinhardtii* (65). In this key respect, COMBO differs from the TAP medium more commonly
438 used in *Chlamydomonas* research (including the Ratcliff, Herron (41) settling selection
439 experiment). TAP contains acetate, which supports heterotrophic growth by *C. reinhardtii*.
440 COMBO necessarily brings about a different physiological state than TAP, and this difference
441 may influence *C. reinhardtii*'s latent tendency to form clonal assemblages and/or extracellular
442 matrices that cause bodies to settle rapidly in aqueous media. Fourth, by luck of the draw we
443 might not have initiated our experiment with the right set of ancestral genotypes, or with
444 sufficient standing genetic variation. Ratcliff, Herron (41) initiated their evolutions using an

445 outbred population that had a high level of standing genetic variation; moreover, all populations
446 went through one round of sexual reproduction (within-population mating) during the evolutions.
447 After 73 transfers (~315 generations) with settling rate selection imposed every three days,
448 multicellular *C. reinhardtii* evolved in only one of 10 replicate populations. The difference
449 between our results and those of Ratcliff, Herron (41) suggests that the amount of standing
450 genetic variation might play a role in the tempo and mode by which multicellularity emerges.
451 Finally, perhaps multicellularity did not evolve under the settling selection here simply because
452 the outcome is stochastic. After all, in Ratcliff, Herron (41) only one of 10 replicate populations
453 evolved multicellularity under settling selection. If we use this as an estimate of the probability
454 of multicellularity evolving under settling selection, the binomial probability of zero of twelve
455 replicates evolving multicellularity would be 0.28 (p(x=0) given 12 trials, each with a probability
456 of success of 0.1), far too likely to rule out.

457 Regarding the genetic basis of multicellularity in our experiments, we only sequenced
458 evolved isolates exhibiting multicellularity at low coverages (14-18X), as our objective was to
459 determine ancestry. Evolved unicellular isolates, in particular ones sharing a common ancestor
460 with the evolved multicellular isolates, were not sequenced due to budget constraints. Therefore,
461 comparisons between unicellular and multicellular evolved isolates, or between founder strains
462 and their evolved multicellular isolates, are not possible for the investigation on the mutational
463 trajectories to multicellularity here. Future directions include repeated backcrossing multicellular
464 traits into the founder or wild-type strains to generate near isogenic lines, or directly sequencing
465 the candidate genes known to cause multicellular phenotypes to identify the mutation(s)
466 responsible for the transitions to multicellularity. For example, previous work has shown that the
467 retinoblastoma (*RB*) cell cycle regulatory pathway is involved in cluster formation in the alga

468 *Gonium*, an undifferentiated colonial relative of *Chlamydomonas* (33). Indeed, expression of the
469 *Gonium RB* gene in a *Chlamydomonas* strain lacking its *RB* gene caused this mutant to form
470 colonial assemblies containing 2-16 cells (33). It is plausible that mutations involved in the cell
471 cycle pathway such as *RB* may underlie the transition from unicellular to multicellular
472 phenotypes in our evolution experiments.

473 Taken together, our findings show that past genetic changes in some strains of
474 *Chlamydomonas* can shift the probabilities of alternative evolutionary paths towards
475 multicellularity. Further, subsequent mutations can play a significant role in shaping divergent
476 multicellular forms even in populations evolved under the same condition.

477

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486

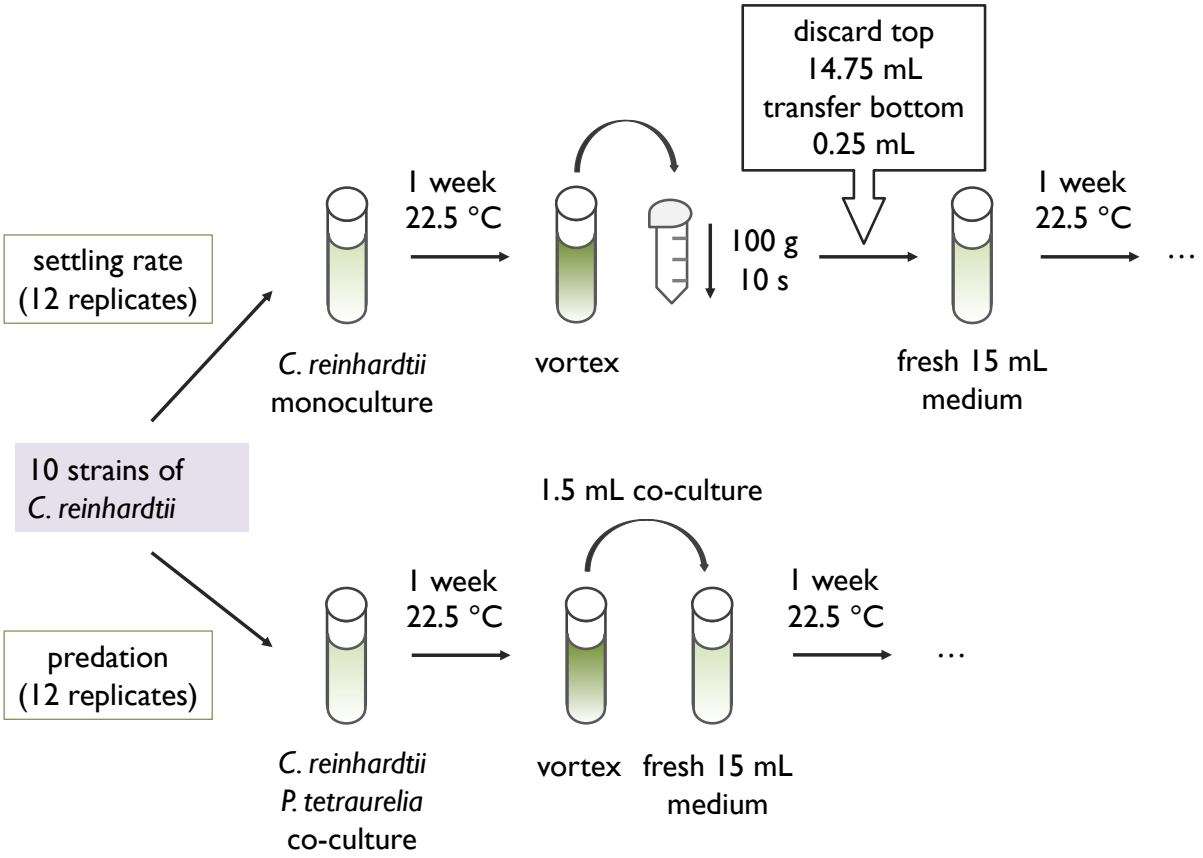

Figure 1. Experimental evolution diagram. A founder population consisting of 10 different strains of *C. reinhardtii*, all in equal proportion, was used to generate 24 replicate populations: 12 were subjected to settling selection that favors large cell clusters that settle rapidly; 12 were subjected to predation by the protist *P. tetraurelia*. Subsamples of each population were transferred to fresh medium every week, with approximately 15 generations elapsing between transfers, for a total of 40 transfers. For the settling rate selection, each population was transferred to a 15 mL Falcon tube and centrifuged at 100 x *g* for 10 s and only the bottom 0.25 mL was transferred to fresh medium. For populations under predation selection, 1.5 mL of co-cultured *C. reinhardtii* and *P. tetraurelia* were transferred to fresh medium every week.

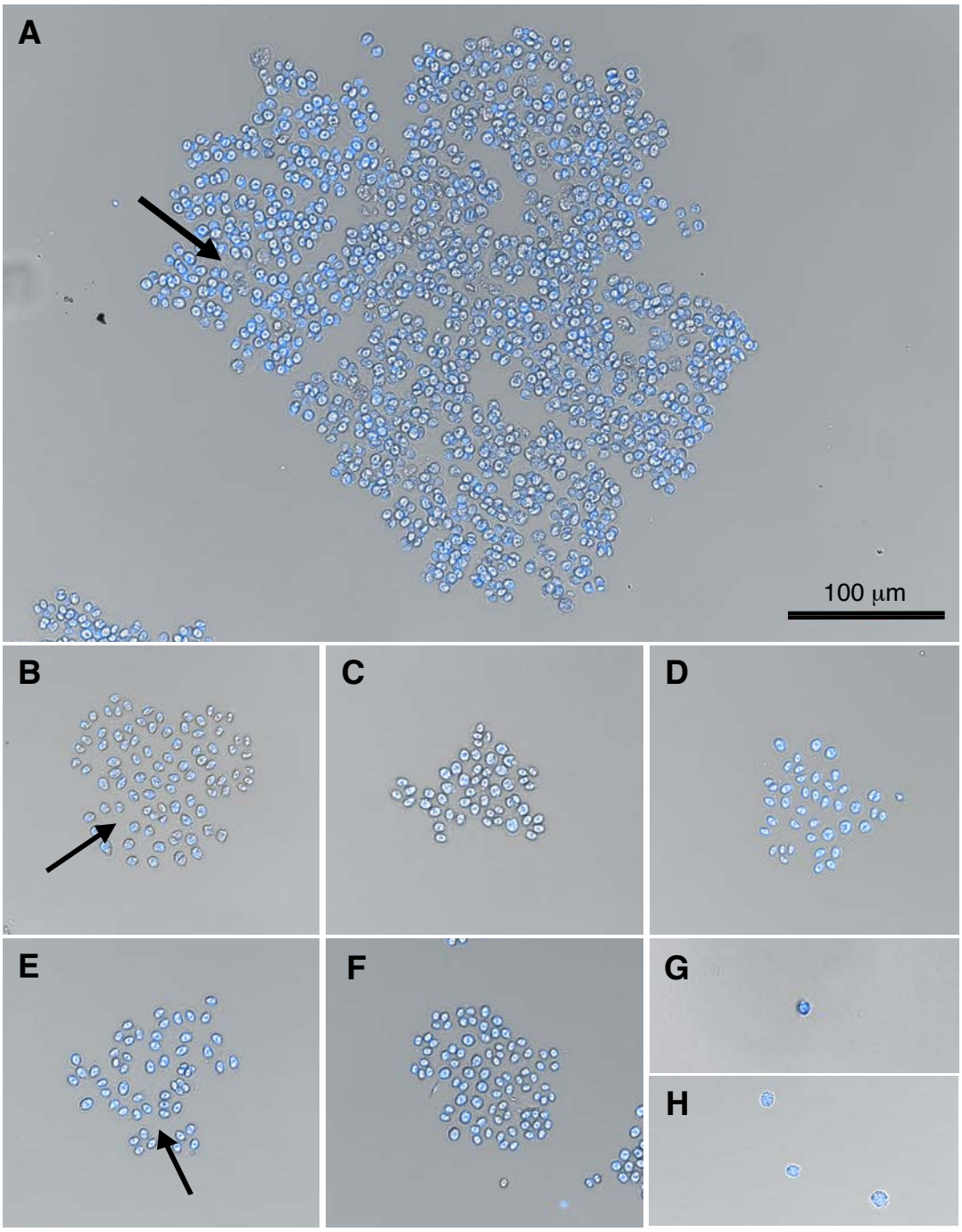

Figure 2. Microscopic images of each evolved strain and their unicellular ancestors stained by DAPI. (A) PS09-32-A; (B) PS11-32-B; (C) PS02-40-A; (D) PS02-40-B; (E) PS02-40-C; (F) PS12-40-C; (G) CC-2936; (H) CC-1690. Black arrows indicate examples of extracellular matrix.

Genomic data indicate that strain PS12-40-C evolved from CC-1690 while the rest of multicellular strains evolved from CC-2936 (see **Table 3**).

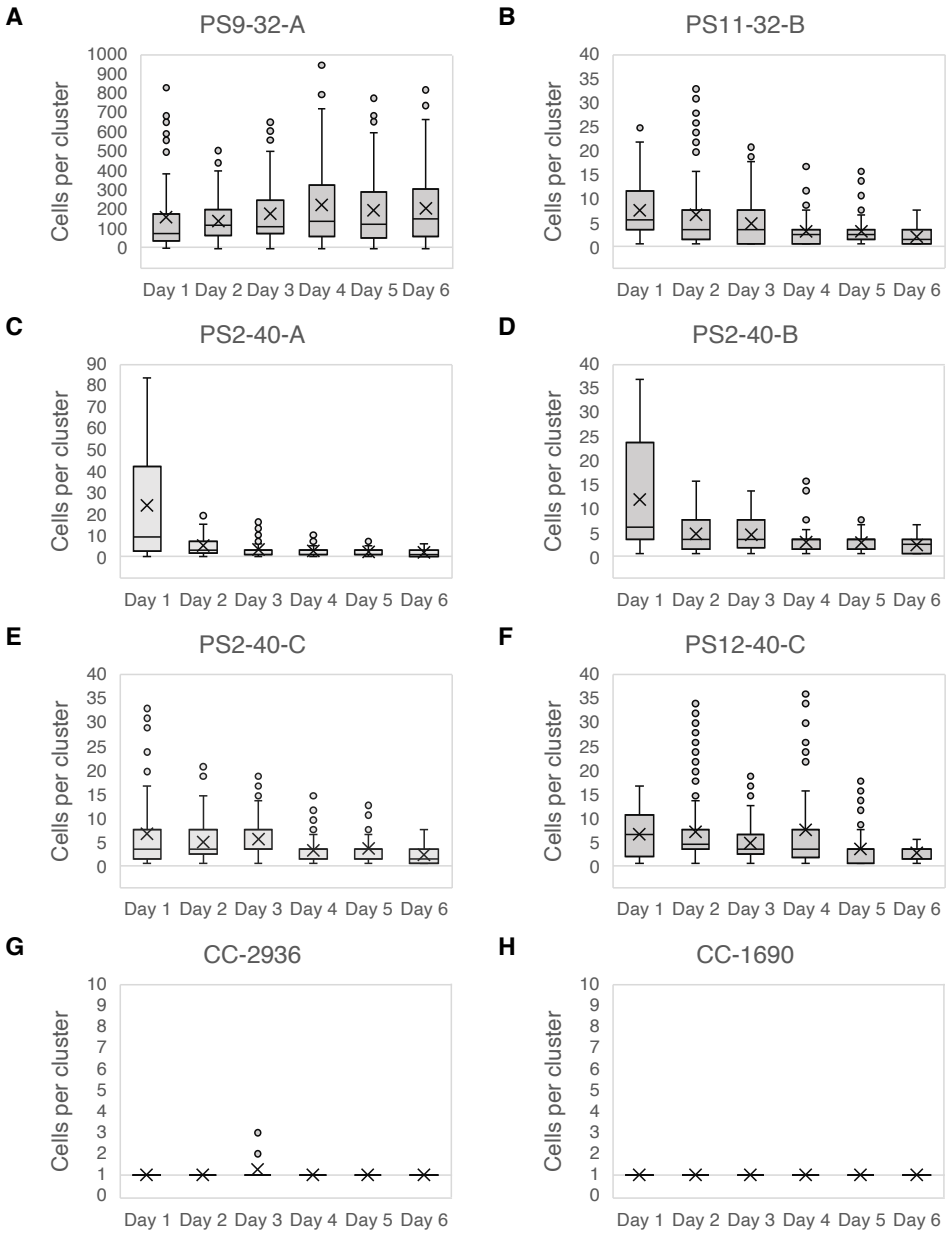

Figure 3. Box plots of cells per cluster over a 6-day period. Cluster sizes were measured by sampling cultures over six days of growth, staining nuclei with DAPI, and imaging using fluorescent microscopy. (A-F) Cluster sizes in the evolved strains; (G-H) Cluster sizes in the ancestors. An x or cross represents the mean of cells per cluster; the crossbar indicates the median of cells per cluster. The grey horizontal bar in each figure represents one cell per cluster. Note the y-axis scales vary among strains.

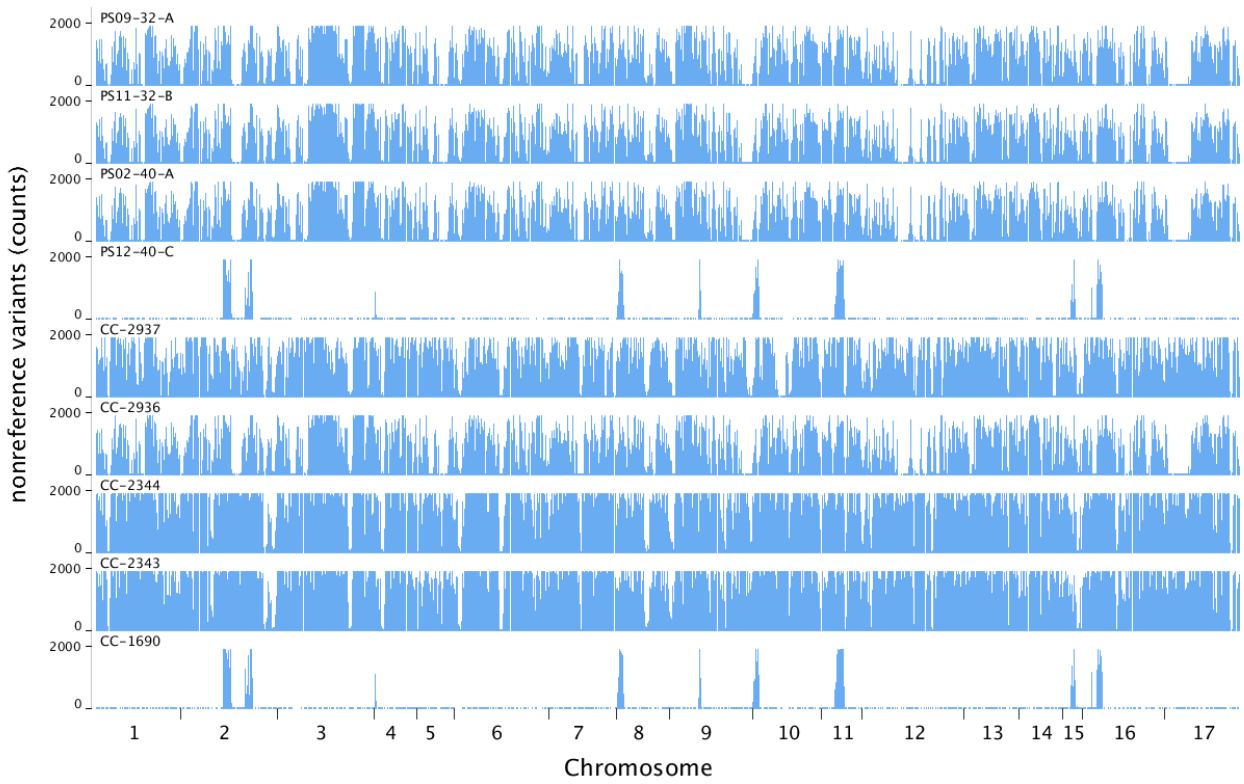

Figure 4. Genome-wide distributions of nonreference variants in evolved and mt+ founder strains on 17 *Chlamydomonas* chromosomes.

Table 1. *C. reinhardtii* founder strains used in this experiment.

Strain	Mating type*	Origin	Location	Reference(s)
CC-1690	mt+	Lab	-	Sager (1955)
CC-5080	mt-	Lab	-	Gallaher et al (2015)
CC-2937	mt+	Field	Quebec, Canada	Sack et al (1994)
CC-2936	mt+	Field	Quebec, Canada	Sack et al (1994)
CC-2935	mt-	Field	Quebec, Canada	Sack et al (1994)
CC-2344	mt+	Field	Ralston, PA	Spanier et al (1992)
CC-2342	mt-	Field	Pittsburgh, PA	Spanier et al (1992)
CC-2343	mt+	Field	Melbourne, FL	Spanier et al (1992)
CC-2931	mt-	Field	Durham, NC	Pröschold et al (2005)
CC-2290	mt-	Field	Minnesota	Gross et al (1988)

*There are two mating types, identical in appearance and known as mating type *plus* and *minus*, in *C. reinhardtii*. The + or - sign in each parenthesis denotes the mating type of each strain. The entry into the sexual cycle is controlled by nitrogen deprivation in *C. reinhardtii*. The media used in the experiment sustain normal cell growth and we expect all reproduction to be asexual.

Table 2. P-values for Dunn's Test for all pairwise comparisons between the maximum cluster sizes in different strains. Single asterisks indicate p-values of <0.05. Double asterisks indicate p-values of < 0.01.

	Maximum cluster size (median)							
	PS09-32-A (156 cells)	PS11-32-B (6 cells)	PS02-40-A (10 cells)	PS02-40-B (7 cells)	PS02-40-C (4 cells)	PS12-40-C (7 cells)	CC-2936 (1 cell)	CC-1690 (1 cell)
PS09-32-A		**	*	**	**	**	**	**
PS11-32-B			0.56	0.81	0.40	0.48	**	**
PS02-40-A				0.72	0.28	0.31	**	**
PS02-40-B					0.38	0.43	**	**
PS02-40-C						0.98	**	**
PS12-40-C							**	**
CC-2936								0.24
CC-1690								

Table 3. Comparisons between genomic variants in evolved and mt+ founder strains. The numbers in parenthesis after strain names indicate the number of nonreference variants identified in each strain (see Methods). Heat maps are color-coded (light grey/low percentage match < grey/middle percentage match < dark grey/high percentage match).

	CC-1690 (90,242)	CC-2937 (1,651,964)	CC-2936 (1,194,250)	CC-2344 (2,102,758)	CC-2343 (2,187,600)
PS09-32-A (1,169,953)	59.56%	35.46%	99.93%	22.50%	18.41%
PS11-32-B (1,110,266)	59.15%	35.11%	99.90%	22.28%	18.18%
PS02-40-A (1,144,326)	59.59%	35.40%	99.93%	22.42%	18.32%
PS12-40-C (77,708)	100%	2.02%	3.58%	1.33%	1.09%

Table 4. Examples of genomic variants that differ among mt+ founder strains and their presence and absence in the evolved strains.

Position \ Strain	CC-1690	CC-2937	CC-2936	CC-2344	CC-2343	PS09-32-A	PS11-32-B	PS02-40-A	PS12-40-C
Chrom_1 1782815	A	T	T	T	T	T	T	T	A
Chrom_1 1796517	A	G	A	A	A	A	A	A	A
Chrom_2 531304	G	G	A	G	G	A	A	A	G
Chrom_2 906332	T	T	T	C	T	T	T	T	T
Chrom_2 1216720	C	C	C	C	T	C	C	C	C
Chrom_3 666196	C	A	T	A	A	T	T	T	C
Chrom_3 983155	AA	AG	GG	GG	AG	GG	GG	GG	AA
Chrom_3 1128242	C	C	T	C	C	T	T	T	C
Chrom_4 14191	TGA	TGA	TGA	TA	TGA	TGA	TGA	TGA	TGA
Chrom_4 236504	G	G	G	G	A	G	G	G	G
Chrom_5 763807	G	A	A	A	A	A	A	A	G
Chrom_5 888052	C	T	C	C	C	C	C	C	C
Chrom_5 1624406	G	G	A	G	G	A	A	A	G
Chrom_6 122423	ACG	ACA	ACA	ATG	ACG	ACA	ACA	ACA	ACG
Chrom_6 204099	T	T	T	T	C	T	T	T	T
Chrom_7 1392564	CCCGC	CC	CC	CC	CC	CC	CC	CC	CCCGC
Chrom_8 301955	TG	AA	TG	TG	TG	TG	TG	TG	TG
Chrom_8 533535	A	A	G	A	A	G	G	G	A
Chrom_9 133015	A	A	A	C	A	A	A	A	A
Chrom_10 69703	C	C	C	C	T	C	C	C	C
Chrom_11 448221	A	C	C	C	C	C	C	C	A
Chrom_13 679947	ATCTAT	AAC	ATCTAT	AAC	AAC	ATCTAT	ATCTAT	ATCTAT	ATCTAT
Chrom_14 153285	G	G	A	G	G	A	A	A	G
Chrom_15 135507	C	C	C	T	C	C	C	C	C
Chrom_16 312391	C	C	C	C	G	C	C	C	C

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