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Phenotypic differences between interfertile *Chlamydomonas* species

We're crossing *C. reinhardtii* and *C. smithii* algae for high-throughput genotype-phenotype mapping. In preparation, we're comparing the parents to uncover unique species-specific phenotypes.

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Purpose

We're working to understand the associations between genotypes and phenotypes across the tree of life. Analyzing variation among interbreeding populations is a powerful tool for dissecting genotype-phenotype relationships.

To build our framework from the bottom up, we're starting by breeding interfertile *Chlamydomonas* species — *C. reinhardtii* and *C. smithii* — and performing high-dimensional characterization of many aspects of their biology. Here, we describe various phenotypes for each of the two parental species. These phenotypes will serve

as a baseline against which we will compare phenotypic and genotypic differences in the progeny of the hybridized strains.

So far, we've studied the two species' growth on various types of media, and expect to have data on their motility, chloroplast fluorescence decay, and organelle morphologies in the near future. We're sharing the data as we collect it, and expect to add new phenotypic data over time. We hope that readers working with these species will find it useful to have access to our findings as soon as we're able to describe them.

- This pub is part of the **platform effort**, "Genetics: Decoding evolutionary drivers across biology." Visit the platform narrative for more background and context.

Background and goals

We are advancing an initiative to understand how genes and environments interact to drive variation in organismal traits. To investigate the relationship between genotypes and phenotypes, we need a population with both genetic and phenotypic diversity. A traditional solution would be to perform mutagenesis in an otherwise isogenic population, and then isolate individual alleles. While useful, the genetic changes that result from mutagenesis are fundamentally different from the forms of genetic variation that drive phenotypic variation in an actively interbreeding natural population. Such an approach also won't allow us to look at variation driven by specific allele combinations. A powerful alternative is to study the relationship between genotypic and phenotypic variation within a naturally varying population, as is done with experiments such as quantitative trait locus (QTL) mapping.

We chose to create a diverse population by hybridizing two interfertile species. We selected *Chlamydomonas reinhardtii* and *Chlamydomonas smithii* because they produce viable progeny when mated **[1][2]**, are easy to maintain in clonal cultures, and, being unicellular algae, offer the potential to perform a variety of high-throughput analyses. However, little is known about the aspects of biology that differentiate these species (see Table 1). Given this, we are comparing a variety of phenotypes among these parent species to identify key axes of variation between them. These analyses will act as priors for all downstream comparisons of their progeny.

Brief background on *Chlamydomonas reinhardtii* and *smithii* species

C. reinhardtii is widely used as a model to study cellular processes like photosynthesis, cilia development, and cell division **[3]**. *C. smithii* is less well characterized, even though these two species share a similar history **[4][5]**. In fact, the cells we used in this study were propagated from cultures isolated from soil fields in Massachusetts in 1945. For more than seven decades, both species have been subcultured under laboratory conditions, implying that any apparent differences between the species shouldn't stem from disparate lab adaptations, since they have been exposed to similar levels of adaptation opportunities.

To date, several genotypic differences have been observed between these two species including differences in their nuclear, chloroplast, and mitochondrial genomes **[6][7][8][9][10]**, but a quality reference genome for *C. smithii* does not yet exist. Phenotypic differences are less well characterized — we've summarized the few previously reported differences in Table 1. The dearth of studies on *C. smithii* has led some to mischaracterize the species as a wild-type *C. reinhardtii* strain. For instance, *C. smithii* was recently used as a baseline wild-type *C. reinhardtii* strain in a comparison with *C. reinhardtii* cell wall mutants **[11]** and has been referred to as a *C. reinhardtii* field strain even though it has been subcultured in labs for the same amount of time as bona fide *C. reinhardtii* lab strains **[6]**.

Phenotype	***C. reinhardtii***	***C. smithii***
Doubling time in the dark **[12]**	~24–31 hours	~72–108 hours
Cell volume **[12]**	110–115 μm^3	100 μm^3
Nuclear volume **[12]**	9.5–10.3 μm^3	11 μm^3
Morphology	10 µm long **[13]** 3 µm wide **[13]** ~6.5 um diameter **[6]**	7.5–11 µm long **[2]** 3–7.5 µm wide **[2]** ~7.5 um diameter **[6]**
Chlorophyll content **[6]**	~1600–2100 APC-A intensity	~1800 APC-A intensity
Heterotrophic growth **[6]**	~0.09-0.27 omnilog units	~0.92 omnilog units
Phototrophic growth **[6]**	—	~0.003 ΔOD_{680}/h

Table 1. Previously reported phenotypic differences between the two *Chlamydomonas* species we're studying.

The approach

Before isolating *C. reinhardtii* × *C. smithii* hybrid strains, it is important that we understand the baseline differences between these two species. We are investigating specific traits in these organisms that we can efficiently adapt to high-throughput methods, align with our research goals, and which take advantage of our in-house tools and expertise. We're using both single-cell and multi-cell analyses to differentiate these species. These findings will provide the foundation for upcoming experiments to map associations between genotypes and phenotypes.

Here, we observed two *Chlamydomonas* species under various growth conditions, altering nutrients, illumination, and temperature. We used two *C. reinhardtii* strains of opposing mating types in this initial study to determine if these phenotypes were mating-type dependent; however, we only used one *C. smithii* strain because a mating type pair of this species is not yet accessible through culture collection centers. We chose which growth media to use in this study based on in-house availability and variability of nutrients. We'll add additional phenotypes to this pub as we generate the data.

Species and strains

Species	Strain numbers and source links	Mating type	Site of parent isolation
Chlamydomonas reinhardtii	137c, cc-124, UTEX2243, SAG 33.89 (source)	(–)	• Amherst, Massachusetts, USA • Potato field soil • 1945
Chlamydomonas reinhardtii	137c, cc-125, UTEX2244, SAG 34.89 (source)	(+)	• Amherst, Massachusetts, USA • Potato field soil • 1945

Species	Strain numbers and source links	Mating type	Site of parent isolation
Chlamydomonas smithii	136f, cc-1373, UTEX1062, SAG 54.72 (source)	(+)	• South Deerfield, Massachusetts, USA • Tobacco field soil • 1945

Table 2. Organisms used in this pub.

We ordered all species from the Chlamydomonas Resource Center.

Cell maintenance

After receiving strains from the culture center (Table 2), we streaked cells out to individual colonies on TAP media with 1.5% agar. We cultured cells from single colonies in liquid TAP media (UTEX) and then seeded them onto plates of TAP media with 1.5% agar to form a confluent lawn either at 24 °C under constant illumination or at ambient temperature under 12:12-hour light:dark cycles.

Extracting data displayed in Table 1

For the chlorophyll content, heterotrophic growth, and phototrophic growth data in Table 1, we extracted raw values from published bar graphs **[6]** using the WebPlotDigitizer tool **[14]**.

Media components

We made all solid media with 1.5% agar. Media components are as follows:

TAP media

375 µM NH_4Cl, 17.5 µM $CaCl_2$*$2H_2O$, 20 µM $MgSO_4$*$7H_2O$, 6 µM Na_2HPO_4, 4 µM KH_2PO_4, 200 µM Trizma base, 170 µM glacial acetic acid, 0.1% v/v of Hunter's trace elements solution (134 µM Na_2EDTA*$2H_2O$, 770 µM $ZnSO_4$*$7H_2O$, 184 µM H_3BO_3, 26 µM $MnCl_2$*$4H_2O$, 18 µM $FeSO_4$*$7H_2O$, 7 µM $CoCl_2$*$6H_2O$, 5 µM $CuSO_4$*$5H_2O$, and 800

nM $(NH_4)_6Mo_7O_{24}$*$4H_2O$). Suspended in fresh water. We purchased liquid media from UTEX.

Bristol media

2.94 mM $NaNO_3$, 0.17 mM $CaCl_2$*$2H_2O$, 0.3 mM $MgSO_4$*$7H_2O$, 0.43 mM K_2HPO_4, 1.29 mM KH_2PO_4, and 0.43 mM NaCl. Suspended in fresh water. We purchased liquid media from UTEX.

Soil extract media

96% Bristol media and 4% pasteurized soil water (1 tsp soil + water) supernatant. Suspended in fresh water. We purchased liquid media from UTEX.

K media

882 µM $NaNO_3$, 50 µM NH_4Cl, 10 µM Na_2 b-glycerophosphate, 504 µM Na_2SiO_3*$9H_2O$, 10 nM H_2SeO_3, 1 mM tris base (pH 7.2), 111 µM Na_2EDTA*$2H_2O$, 12 µM $FeCl_3$*$6H_2O$, 900 nM $MnCl_2$*$4H_2O$, 80 nM $ZnSO_4$*$7H_2O$, 42 nM $CoCl_2$*$6H_2O$, 26 nM Na_2MoO_4*$2H_2O$, 10 nM $CuSO_4$*$5H_2O$, 296 nM thiamine HCl, 0.21 nM biotin, and 0.04 nM cyanocobalamin. Suspended in filtered synthetic seawater (RICCA Chemical Company: R8363000) [water, sodium chloride, magnesium chloride hexahydrate, sodium sulfate anhydrous, calcium chloride dihydrate, potassium chloride, sodium bicarbonate, potassium bromide, strontium chloride hexahydrate, boric acid, sodium hydroxide, and sodium fluoride]. We purchased a K media kit from the NCMA at Bigelow Labs.

L1 media in marine broth (MB + L1)

882 µM $NaNO_3$, 36.2 µM NaH_2PO_4*H_2O, 106 µM Na_2SiO_3*$9H_2O$, 11.7 µM Na_2EDTA *$2H_2O$, 11.7 µM $FeCl_3$*$6H_2O$, 900 nM $MnCl_2$*$4H_2O$, 80 nM $ZnSO_4$*$7H_2O$, 50 nM $CoCl_2$*$6H_2O$, 1 nM $CuSO_4$*$5H_2O$, 8.22 nM Na_2MoO_4*$2H_2O$, 1 nM H_2SeO_3, 1 nM Na_3VO_4, 1 nM K_2CrO_4, 296 nM thiamine HCl, 0.21 nM biotin, and 0.04 nM cyanocobalamin. Suspended in Marine Broth 2216 (Sigma-Aldrich) [water, peptone, yeast extract, ferric citrate, sodium chloride, magnesium chloride, magnesium sulfate, calcium chloride, potassium chloride, sodium bicarbonate, potassium bromide, strontium chloride, boric acid, sodium silicate, sodium fluoride, ammonium nitrate, and disodium phosphate]. We purchased an L1 media kit from the NCMA at Bigelow Labs. In this instance, we suspended L1 in Marine Broth 2216 instead of synthetic seawater because autoclaving

synthetic seawater often results in nutrients precipitating out **[15]** and the addition of peptone increases the media's buffer capacity, preventing excessive precipitation **[16]**.

F/2 media

880 µM $NaNO_3$, 36 µM NaH_2PO_4*H_2O, 106 µM Na_2SiO_3*$9H_2O$, 11.7 nM Na_2EDTA*$2H_2O$, 11.7 nM $Fe(NH_4)_2(SO_4)_2$*$6H_2O$, 0.9 nM $MnSO_4$*H_2O, 0.08 nM $ZnSO_4$*$7H_2O$, 0.05 nM $CoSO_4$*$7H_2O$, 0.04 nM $CuCl_2$*$2H_2O$, 0.03 nM Na_2MoO_4*$2H_2O$, 100 nM cyanocobalamin, 126 uM thiamine, and 100 nM biotin. Suspended in pasteurized seawater. We purchased liquid media from UTEX.

F/2 – Si media

882 µM $NaNO_3$, 36.8 µM NaH_2PO_4, 11.7 µM Na_2EDTA*$2H_2O$, 11.6 µM $FeCl_3$*$6H_2O$, 909 nM $MnCl_2$*$_4H_2O$, 76 nM $ZnSO_4$*$7H_2O$, 42 nM $CoCl_2$*$2H_2O$, 40 nM $CuSO_4$*$5H_2O$, 25 nM Na_2MoO_4*$2H_2O$, 20.5 nM biotin, 4 nM cyanocobalamin, and 296 nM thiamine. We purchased liquid media from Sigma-Aldrich (G0154) at 50× concentration and diluted to 1× in filtered synthetic seawater (RICCA Chemical Company: R8363000) [water, sodium chloride, magnesium chloride hexahydrate, sodium sulfate anhydrous, calcium chloride dihydrate, potassium chloride, sodium bicarbonate, potassium bromide, strontium chloride hexahydrate, boric acid, sodium hydroxide, and sodium fluoride].

Spot assays

We maintained cells on plates of TAP media with 1.5% agar under a 12/12-hr light-dark cycle at ambient temperature. One day prior to experimentation, we seeded cells in 3 mL of liquid TAP media or sterile water and grew them at ambient temperature under a 12/12-hr light-dark cycle while rotating at 46 rpm at a ~120° angle in a rotator drum. The next day, we measured the optical density at 730 nm for each strain using a SpectraMax iD3 plate reader (Molecular Devices). We diluted dense cultures to have equal concentrations of cells. We conducted a serial dilution of the samples, following a progressive pattern of (1:1, 1:2, 1:4, 1:8, 1:16) to gradually reduce the concentration at each successive step In either water or TAP. We spotted samples grown under each condition onto nine agar plates per growth medium in 10 µL volumes. We kept plates at ambient temperature overnight to allow spots to dry.

The next day, we flipped and sealed plates. We then grew three plates from each group at 17 °C or ambient temperature under a 12/12-hr light-dark cycle or at 24 °C under

constant illumination. We allowed cells to grow for 16 days and then imaged the plates using an Azure 600 imaging system (Azure Biosystems) under “true color imaging” settings.

Microscopy

Sample prep

We grew cells on 1.5% agar in the indicated medium. We picked individual colonies with a sterile loop and suspended them in sterile water, placed in a 96-well plate with a #1.5 glass coverslip, and imaged immediately. For Video 1 and Video 2, we suspended cells in L1 media and plated on an Arralyze glass coverslip with 400 µm flat-bottom wells. We mounted coverslips on a standard microscope slide and sealed with VALAP (1:1:1 mixture of Vaseline, lanolin, and paraffin wax).

Imaging

We acquired micrographs with a Yokogawa CSU W1-SoRa scanner unit attached to a Nikon Ti2-E confocal microscope set up with a LIDA Light Engine for RGB color images. We used a Plan Apo λ 60× oil objective with a 1.5× magnifier or a Plan Apo λ 10× air objective. We processed all imaging data using FIJI software (NIH). We used the StackReg Fiji plugin from the BIG-EPFL package to correct for sample drift during extended time-lapse imaging **[17]**.

Writing

We used ChatGPT to suggest wording ideas and streamline/clarify content, and then edited the AI-generated text.

The results

We can maintain both *C. reinhardtii* and *C. smithii* in standard TAP (tris-acetate-phosphate) media **[12]**. However, we were surprised to see *C. smithii* outgrowing *C. reinhardtii* strains on other Chlorophyceae media — Bristol media and soil extract media (Figure 1). Bristol media contains more than 5× the amount of nitrogen and 300× the amount of phosphate compared to TAP media. Soil extract media is Bristol

media supplemented with 4% pasteurized soil, which likely contains heavy metals similar to TAP media, suggesting *C. smithii* is more adaptable to high-nutrient media than *C. reinhardtii*. The temperature and illumination conditions had no obvious impact on colony growth, except visible colonies started to appear quicker under constant illumination in a controlled 24 °C environment, consistent with previous work **[18]**.

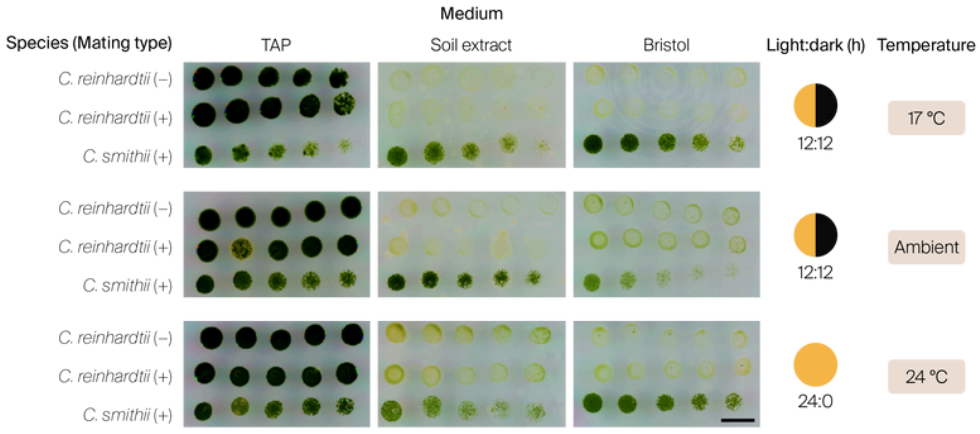

Figure 1

Both species grow on TAP media, but *Chlamydomonas smithii* can also grow on soil extract and Bristol media.

Representative spot assays of *C. reinhardtii* (cc-124 and cc-125) and *C. smithii* (cc-1373) on TAP (tris-acetate-phosphate), soil extract, or Bristol media under different growth conditions. Scale bar: 10 mm.

Since *C. smithii* was able to grow on richer media that includes ~0.4 mM sodium chloride, we wondered if *C. smithii* could grow on high-salt marine media. We spotted the three *Chlamydomonas* cultures onto four different solid marine media that are often used to grow diatoms and other marine protists, which we already had in house **[19][20][21]**. Unsurprisingly, we did not observe any growth on K media, F/2 media, or F/2 lacking silica (Figure 2). However, to our surprise, we observed slow, limited growth of *C. smithii* on marine broth media with L1 nutrients containing more than 8,000× more sodium chloride than Bristol media (Figure 2). This growth was apparent under all illumination and temperature conditions but much more prominent when grown under constant illumination at 24 °C, as expected. Since K media and L1 media are very similar (albeit for the tris and ammonium chloride in K media), we suspect that the presence of rich marine broth containing peptone and yeast extract might be responsible for the growth of *C. smithii*, consistent with reports that *C. smithii* shows

more heterotrophic growth than *C. reinhardtii* strains **[6]**. We are currently testing this hypothesis and hope to include the results in future iterations of this pub.

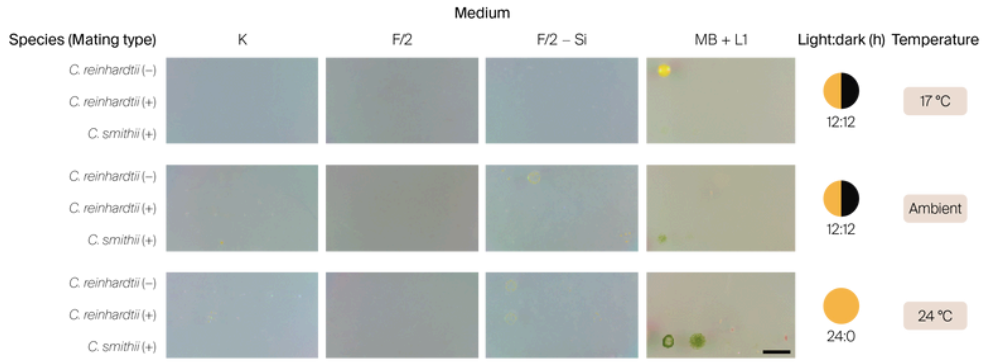

Figure 2

Neither species grows much on salt water media, but *C. smithii* can grow slowly on marine broth with L1 nutrients included.

Representative spot assays of *C. reinhardtii* (cc-124 and cc-125) and *C. smithii* (cc-1373) on K media, F/2 media, F/2 lacking silica, or marine broth (MB) with L1 nutrients under different growth conditions. Yellow growth on the top-right MB + L1 image is a contaminant. Scale bar: 10 mm.

We were surprised to observe *C. smithii* surviving on high-salt marine media and wanted to take a closer look to see if there were any morphological phenotypes under these hypersalinic conditions. Interestingly, when we picked these cells from plates with MB + L1 in 1.5% agar and resuspended them in fresh water, the cells were much larger and round (Figure 3).

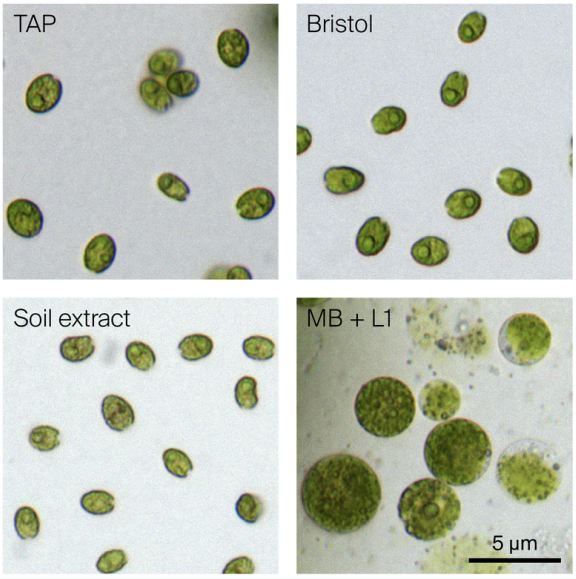

Figure 3

***C. smithii* cells become large and round on MB + L1 media**.

Representative color images of *C. smithii* grown on the depicted media and resuspended in water. TAP: tris-acetate-phosphate; MB + L1: marine broth with L1 nutrients added.

This phenotype was also present when we took cells from MB + L1 plates and suspended them in liquid L1 media. However, in this case, we noticed several large cells with oblong morphologies that quickly rounded up after intense light exposure on the microscope (Video 1). These cells retained their green pigmentation and internal dynamics throughout the experiment, suggesting the cells are alive (Video 2). Surprisingly, we were unable to observe cell division occurring over a 67-hour period. We streaked these cells back onto TAP media and they returned to their normal morphology, confirming that this observation was a result of growth in the MB + L1 media and not contamination. *C. reinhardtii* did not grow on any marine media so we did not observe this morphology in either *C. reinhardtii* strain.

Video 1

***C. smithii* cells becoming more round upon intense light exposure**.

We grew *Chlamydomonas smithii* cells on 1.5% agar plates supplemented with L1 nutrients and marine broth. We imaged isolates in 400 µm glass wells in L1 media (lacking silica) at 30 minutes/frame.

Video 2

Amorphous *C. smithii* cells retain healthy color before and after morphological changes.

We grew *Chlamydomonas smithii* cells on 1.5% agar plates supplemented with L1 nutrients and marine broth. We imaged isolates in 400 µm glass wells in L1 media (lacking silica) at 30 minutes/frame. We captured initial and final frames in RGB, while the intermediate frames were in grayscale.

Key takeaways

Chlamydomonas reinhardtii and *smithii* were isolated in the same year under similar conditions and maintained through continuous subculturing, but, we have observed clear differences between the two species:

- *C. reinhardtii* and *C. smithii* grow well on TAP media at 24 °C under constant illumination.
- *C. smithii* is more adaptable to various growth media.
- *C. smithii* takes on a unique morphology when grown on high-salt marine media.

Next steps

Our ultimate goal is to find a set of traits that differ quantitatively between *C. reinhardtii* and *C. smithii* so that we can compare the phenotypes of their progeny back to the parent species. We're happy with these initial results, but we're looking into other phenotypes as well, including organelle morphology (using live, super-resolution fluorescence microscopy), growth curves in liquid media, chloroplast fluorescence decay, cell wall integrity, individual cell motility behaviors, flagellar beating rates, and phototaxis assays. Please let us know if there are any other phenotypes that you think would be worth exploring!

In addition to identifying useful phenotypes, we're developing novel techniques to quantify them in a high-quality, high-throughput manner. We're also working on high-throughput genotyping methods so that we can thoroughly map genotype-phenotype linkages of the recombinant progeny.

We're excited to use this pub as a test case for modular publishing and will continually update the text and figures to reflect new observations and findings, so stay tuned!

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