Chapter 18

Studying Whole-Genome Duplication Using Experimental Evolution of *Chlamydomonas*

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Abstract

In this chapter, we present the use of *Chlamydomonas reinhardtii* in experiments designed to study the evolutionary impacts of whole genome duplication. We shortly introduce the algal species and depict why it is an excellent model for experimental evolution. Subsequently, we discuss the most relevant steps and methods in the design of a ploidy-related *Chlamydomonas* experiment. These steps include strain selection, ploidy determination, different methods of making diplo- and polyploid *Chlamydomonas* cells, replication, culturing conditions, preservation, and the ways to quantify phenotypic and genotypic change.

Key words *Chlamydomonas reinhardtii*, Experimental evolution, Polyploidy, Whole-genome duplication, Evolve and resequence (E&R)

1 Introduction

"A harnessed soil dwelling animal with multiple stomachs, a single small eye and a filamentous proboscis" (Fig. 1). Ehrenberg's [1] description of *Chlamydomonas* seems to come straight out of a medieval bestiary. While nineteenth-century *Chlamydomonas* was little more than a curiosity for naturalists to observe with early microscopes, these first observations set the stage for the rise of *Chlamydomonas* in the twentieth century as a model for genetics. Pascher [2] pioneered "chlamygenetics" by crossing morphologically different *Chlamydomonas* species and demonstrating Mendelian segregation in the four meiospores derived from a single zygote [3]. But the use of *Chlamydomonas* really took off with Moewus' disputed claims of relative sexuality in *Chlamydomonas* (e.g., [4]), marking the start of microbial biochemical genetics [5, 6]. He was soon followed by others such as Smith (e.g., [7]), Lewin (e.g., [8]), and Sager (e.g., [9, 10]). Within the North-American community, Smith's *Chlamydomonas reinhardtii* isolates soon became the

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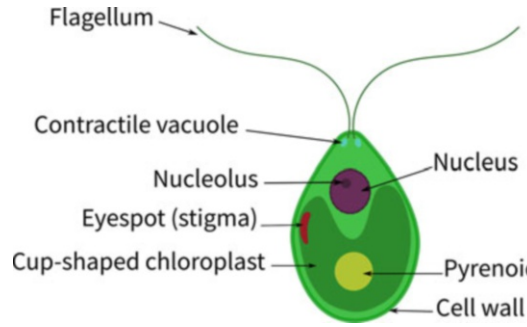

Fig. 1 Labeled diagram of a *Chlamydomonas reinhardtii* cell

preeminent model system. Factors contributing to their establishment were a high growth rate under laboratory conditions, a well-known life cycle that could be fully completed on a chemically defined medium, the ability to grow in the absence of light, and easily inducible sexual reproduction [11]. Experimental work on *Chlamydomonas* laid the foundations for our understanding of photosynthesis, the eukaryotic flagellum (cilium), basal bodies, and more [12]. Moreover, the genome of *C. reinhardtii* was one of the first algal genomes sequenced [13] and is currently one of the eight flagship JGI plant genomes[1]. Consequently, *Chlamydomonas* is probably the best studied green cell and a pivotal model organism whose importance transcends the plant kingdom and has even been dubbed as the green yeast [14].

Apart from its importance for physiology and genetics, the fast growth rate with a doubling time of 8 hours [15], the ease of culturing both sexually and asexually, and the low space requirements make *C. reinhardtii* an excellent system to study evolution. Bell and colleagues [16–21] were some of the first to realize this and, to the best of our knowledge, designed the first experimental evolution with *Chlamydomonas* [22–24]. During the past three decades, *C. reinhardtii* has been used to investigate a wide variety of evolutionary topics related to sex [25–27], population size [28], epigenetics [29], evolutionary rescue [30, 31], adaptation to different rates of environmental change [32], and the emergence of multicellularity [33, 34]. In this chapter, we present methods and considerations that will allow the use of *C. reinhardtii* to study shifts in ploidy and their importance for evolution.

Ploidy shifts, especially whole-genome duplications (WGD), are a prominent phenomenon and are often considered as a major force in evolution. WGDs are thought to alter the rate of adaptation and mutation [35], and the redundancy of the extra genome copy is believed to increase robustness and evolvability by allowing the accumulation of mutations that might eventually cause neo-

[1] https://jgi.doe.gov/our-science/science-programs/plant-genomics/plant-flagship-genomes/

and subfunctionalization [36–40]. Although these hypotheses are largely supported by observation and simulations, experimental work is crucial to test their validity. So far, the yeast species *Saccharomyces cerevisiae* has been the main model for experiments considering the evolutionary effects of polyploidization (e.g., [41–48]). Yet, although polyploidy resulting from WGD has been observed in animals and fungi, WGD has been most prevalent in plants and the so-called green lineage. Therefore, to fully understand the mechanistic underpinnings of polyploidy as well as the impact of WGD on evolution, we believe additional experimental evidence, preferably from plants (*see* Chap. 19) or plant-like organisms (this chapter) is needed.

2 Selecting the Experimental Strains

Testing effects of genome copy numbers requires organisms of different ploidy levels. *C. reinhardtii* (further referred to as *Chlamydomonas*) cells are haploid for most of their active life, although they shortly go through a diploid phase during sexual reproduction where two cells of opposite mating-type fuse to form a diploid zygote. Nevertheless, vegetative diploids can be found [49]. They are formed as a result of a defect during sexual reproduction where a small proportion of the gametic fusion products fails to form thick-walled resting cells (i.e., the zygospores) and continue to grow mitotically. *Chlamydomonas* is a heterothallic species, and as all vegetative diploids have the same mating type (*mt-* is dominant), they cannot form vegetative tetraploids or stress resistant zygospores, reducing their long-term survival chances. Luckily, there are a couple of methods that allow to create artificial diplo- and polyploids (discussed in detail in Subheadings 4 and 5). Even though some diploid strains can be ordered from culture collections such as the *Chlamydomonas* Resource Center[2] and are readily available in many labs, polyploids come in many shapes and sizes, and we would advise to put some time in creating and/or selecting strains that are well suited for your experiments.

Polyploids can be autopolyploid or allopolyploid. Traditionally, this classification was binary and based either on the origin (intraspecific for autopolyploids, interspecific for allopolyploids) or on cytological criteria (multivalent pairing in autopolyploids and bivalent pairing for allopolyploids). The existence of segmental allopolyploids, interracial autopolyploids, and a "speciation gray-zone" [50] makes these traditional categories difficult to work with [51–54] and supports the notion of a continuous spectrum starting from pure autopolyploidy with virtually identical genomes going

[2] https://www.chlamycollection.org/

over interracial autopolyploids and segmental allopolyploids toward allopolyploids with stronger genetic divergence. We here use the term "autopolyploid" for full autopolyploids that are solely affected by the effects of genome doubling and allopolyploid for organism that are subjected to the effects of heterozygosity and hybridization as well. An additional factor that should be considered when creating polyploids or even when selecting haploids is the genotype and phenotype of the strain(s) itself. Genotype-wise *Chlamydomonas* is one of the most diverse eukaryotic species. Its nucleotide diversity at synonymous sites is approximately 3% [55]. The 36 field isolates with sequenced genomes (deposited in either the *Chlamydomonas* resource center or the NIES culture collection) can be subdivided in three geographical clusters [56], making *Chlamydomonas* exceptionally well suited to study the effects of allopolyploidization. Phenotypical variation is equally great and some strains might be more suitable for experimental manipulation than others. The list of wishful features depends on the experiment, but in general, a good experimental strain does not grow in palmelloids (i.e., clumps of nonflagellated cells) that hinder phenotyping and cell counts and have an intact circadian clock mechanism (important for synchronization) and a high mating efficiency (especially when crosses are needed to create polyploids). Unfortunately, the use of standard laboratory strains does not guarantee that these prerequisites are met.

All laboratory strains trace back to a single zygote colony obtained by Gilbert Smith from soil collected on a potato field in Amherst, Massachusetts, in 1945 [11]. Molecular evidence has confirmed that all strains contain material from two haplotypes but that the contribution of both parents is not equal. It is now generally accepted that Gilbert Smith performed several backcrosses before distributing the five strains that became the ancestors of all contemporary lab strains to other laboratories [57]. Currently, some laboratory strains are available in isogenic pairs (differing only in mating type), but the most divergent strains have a maximum of 25% of their genomes coming from different ancestral haplotypes (thanks to the unequal contribution of both parents in the strains distributed by Smith). Despite their common ancestry, laboratory strains can show considerable phenotypical differences [57].

3 Determining Ploidy Level

When working with strains of different ploidy, ploidy determination is common practice. It can serve as an early indicator of strain contamination or mislabeling and is indispensable when making polyploids (see Subheadings 4 and 5).

A first method to determine ploidy takes advantage of known differences between the genome copies present. If such differences exist and are known, the ploidy can be assessed either phenotypically, for example, when the difference consists of auxotrophic mutations or by using molecular techniques such as a simple chelex DNA extraction [58] followed by mating-type PCR [59, 60] that is commonly used on natural diploids in which the mating-type locus differs between both genome copies. Although very useful, the method is not entirely foolproof, when the genome size is reduced (i.e., diploidization/haploidization), this cannot be detected as long as the mating-type loci remain present in their original copy number.

Determining DNA content through flow cytometry, a second method, is the golden standard for ploidy determination in *Chlamydomonas* and organisms alike. Because DNA content of *Chlamydomonas* cells varies during the cell cycle, it is important to use synchronized cultures or even gametes. Synchronization can be obtained by growing the cells in acetate-free medium under an alternating light-dark regime (usually 12:12, see [61]). Under these settings, cells increase in size during the light period and divide mitotically in the dark, while the number of divisions depends on the size at the end of the light phase [62]. Cells collected at the end of the dark phase (in the early G1 phase) are ideal for flow cytometry. Gametes can be produced by harvesting cultures at a cell density of 0.5 to 1 million cells ml^{-1} (by eye, the cultures are light green), resuspending them in nitrogen-free medium, phosphate buffer, or even distilled water, and incubating them agitated (shaking or bubbling) for 8 to 24 hours at 20–25 °C under 50–100 PPFD (μmol m^{-2} s^{-1}). After harvesting, the cells can be fixed in a 3:1 EtOH:Acetic acid mix for 15 min and kept at −20 °C for longer storage. Before staining, the cell should be washed twice with PBS and treated with RNAse (100 μg ml^{-1}) for 1 hour at 37 °C. PI and Sybr-green can penetrate the cell wall, but DAPI cannot. Consequently, the cultures should be treated by a cell wall resolving solution such as the commercially available enzyme subtilisin [63] or autolysin. Autolysin can be produced by bringing gametes of both mating types together and centrifugation to remove the cells when the majority of cells is quadriflagellate (30 min-1 hour). Autolysin can be stored at −80 °C and should be filter-sterilized before use [64].

A third method to determine ploidy is fluorometric-based quantification of DNA extracted from a known number of cells. DNA should be extracted using a method that produces a high yield, such as traditional cetrimonium bromide (CTAB) and phenol-chloroform extractions or genomic DNA extraction kits, while Qubit (Invitrogen, Carlsbad, CA, USA) can be used for fluorometry. This method was, for example, used by Kariyawasam et al. [65], and the quantification of the DNA from our own

extractions seems to support the accuracy of this method reasonably well. Nevertheless, we would recommend treating the results with care. There might be differences in extraction efficiency between strains or ploidy levels, the efficiency could be restricted to a certain range of starting material quantities, and differences in pipetting – especially during the phase separation – will influence the results. A phase separating gel can be used to address this last problem [65].

Finally, cell size can be used as an indicator of ploidy, but since this character is highly variable both between [55, 57] and within strains depending on the growth conditions, the use of cell size as a ploidy-proxy should only be considered for strains of which the cell size and cell size plasticity are well known and should be combined with another method preferentially flow cytometry. As for flow cytometry, the problem of cell cycle heterogeneity can be overcome by using either synchronized cultures or gametes.

4 Creating Allopolyploid Strains

There are two methods that allow the creation of diplo-/polyploid cells containing divergent genomes. The first one is the laboratory version of the natural diploid formation discussed above. First, two strains of opposing mating type are selected; these strains should be fully interfertile and preferably have a high mating efficiency. If the mating type is unknown, it can be assessed via PCR of the mating-type locus or by crossing with a strain of known mating type. Once the strains are cultivated and gametogenesis is induced (see above), the gametes are mixed and left steady for 60–90 minutes to allow cell fusion. Subsequently the mating products are plated on agar plates, and the vegetative diploids are selected. The resulting agar plates will contain a mix of parental cells, vegetative diploids, and zygotes. Because vegetative diploids are formed at a low frequency, selection of diploids is often facilitated by using parental strains bearing mutations with easily assessable phenotypical effects such as auxotrophic or antibiotic resistance mutations in combination with selective medium; for instance, an arginine requiring *arg7-2* mutant can be crossed with an arginine requiring *arg2* mutant. This way the parents cannot grow on the selective agar plate (here without arginine), and because zygospore maturation, meiosis, and hatching take several days, the vegetative diploids will be the only colony forming cells present (Figs. 2 and 3). For more examples of these (double) complementation schemes, see [66–70]. When strains of interest do not have any useful marker mutations, transformation with an antibiotic resistance gene might be an option [9, 71–73]. Because mutations in these markers are not uncommon and early hatching zygotes can produce viable offspring due to recombination, ploidy should always be verified using a mating-

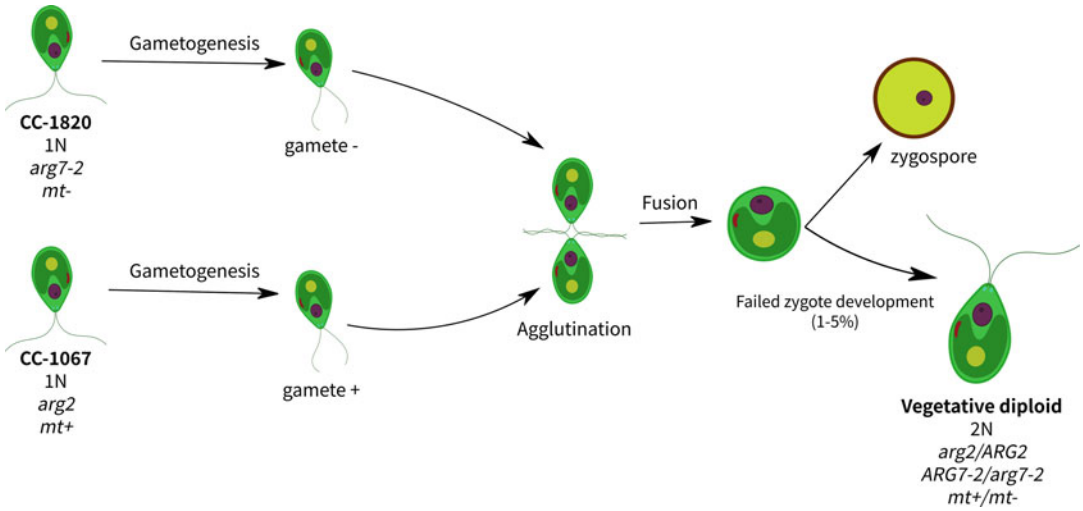

Fig. 2 Sexual cell fusion: example of double complementation scheme with two arginine-requiring strains. Haploid parents CC-1820 and CC-1067 have an auxotrophic mutation that prevents growth in absence of arginine. However, when both haploid genomes are merged in a single nucleus during mating and subsequent failed zygote development, both markers complement and the diploid can grow in a medium without arginine, allowing isolation of the vegetative diploid

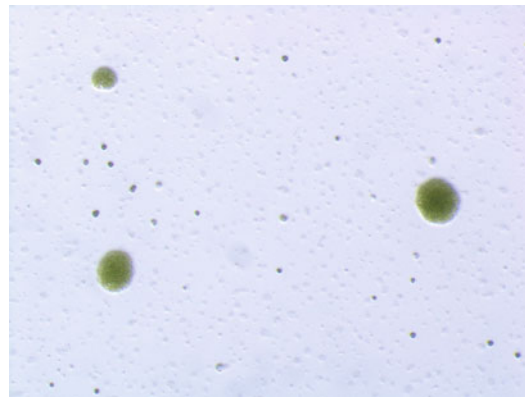

Fig. 3 Vegetative diploid colonies growing on selective medium after double complementation mating

type PCR or flow cytometry. Although the mating-type dominance makes it challenging to produce anything but diploids using this method, it is not impossible because homozygous *mt+* diploids can be generated using a different method (e.g., PEG-fusion, see below). Additionally, there is a mutation that creates a loophole in the genetic makeup of the heterothally that allows to produce diploid gametes behaving as *mt+* [65, 74]. The responsible mutation, *iso1*, discovered by Campbell et al. in 1995 [75], comes only to expression during gametogenesis in *mt-* strains and results in a mixture of regular *mt-* and pseudo *mt+* gametes. In haploids, *iso1*

expression results in isoagglutination but no fusion which requires the *mt+* specific *FUS1* gene. But as vegetative diploids with the *iso1* mutation do have a *FUS1* gene, they do allow homothallic reproduction resulting in *mt+/mt+* and *mt-/mt-* offspring. These can subsequently be used to obtain tetraploids and even higher levels of polyploidy [65]. When applying this method, it should be noted that, although *iso1* is dominant, there seems to be epigenetic silencing over time, and consequently it might be necessary to go through a sexual cycle to reactivate the gene [65].

A second method to bring divergent genomes together in a single cell is polyethylene glycol (PEG)-induced protoplast fusion. As the name suggests, it is also based on cell fusion, but in contrast to the previous method, it does not rely on the sexual cycle and as such can be used to merge cells that are not sexually compatible. This opens up opportunities to generate diploids that are homozygous for one mating type and to make interspecific crosses. For instance, Fowke et al. [76] used this method to fuse *Chlamydomonas* and carrot (*Daucus carota*) cells. PEG-fusion was developed in the 1970s to allow the production of hybrid cells that could not be obtained by crosses (e.g., [77]). The exact mechanism is not entirely understood, but it is believed that the hydrophilic polymer induces agglutinations and increases cell-to-cell contact by making the presence of water between the cell membranes thermodynamically unfavorable [78, 79]. *Chlamydomonas* PEG-fusion was pioneered by Matagne [80, 81] for cell wall-deficient mutants and by Galloway and Holden [82] for regular strains, and their protocols are still valid today. Selection of fusion products can again be facilitated by using a cell sorter or using markers as described above. Although there are no reports on unstable *Chlamydomonas-Chlamydomonas* fusion products, it should be noted that instability has been reported in other organisms such as yeast [83] and that the nuclear material of *Chlamydomonas* disappeared fast in Fowkes' [76] *Chlamydomonas*-carrot fusion products.

Although all these methods allow the production of stable vegetative diploids, the stability of higher ploidy strains obtained using this method remains an open question. And as artificial polyploids in other unicellular eukaryotes such as yeasts are known to fall back to their ancestral ploidy level [44], the stability of all diploid or polyploid lines should be tested by regularly checking the ploidy of these lines (see Subheading 3).

5 Creating Autopolyploid Strains

When fully autopolyploid strains are required, one can use colchicine or some other mitotic inhibitor such as oryzalin and amiprophos-methyl (APM). Colchicine, an alkaloid that naturally occurs in the plant *Colchicum autumnale*, binds to the β-tubulin

units in the microtubules, where it has a destabilizing and depolymerizing effect. During mitosis, microtubule formation is blocked, which hinders separation of the chromosome pairs, causing failure of cytokinesis and metaphase cell cycle arrest [84]. Colchicine is considered the most reliable chemical to induce chromosome doublings in plants and has been used extensively to establish higher ploidy in plant and macroalgal lines since the mid-1930s [85, 86]. Inspired by the early successes with plants, the first attempts to polyploidize *Chlamydomonas* cells followed shortly thereafter [87–89]. There are conflicting results on the efficiency of colchicine to increase ploidy in *Chlamydomonas*. Wetherel and Krauss [90] were the first to obtain stable diploid lines of *C. reinhardtii* after exposure of 8 hours to 10 g L^{-1} colchicine. However, it is unclear how long these *Chlamydomonas* lines remained polyploid. Subsequent experiments (e.g., [91–93]) are less positive and report that the cells that do recover eventually fall back to their haploid size. Nevertheless Walne [92] reported the presence of some larger cells 5 days after transfer to regular medium; consequently, it is not unthinkable that colchicine treatment can induce autopolyploidy in *Chlamydomonas*, although probably at way lower efficiency than in land plants. Based on our own experience, we recommend exposing the cultures to 2 g L^{-1} colchicine for 24 hours. Lower incubation times produce less polyploid cells that return faster to their original ploidy. When using shorter exposure times, one should verify that the exposure interval includes the short (30–40 minutes) S/M phase of the cell cycle during which the genetic material is doubled and mitosis occurs (for information on the cell cycle, see [62]).

Alternatively, the methods described above for creating strains with divergent genome copies can be adopted. PEG-fusion can be applied to fuse cells from a single strain. The absence of markers to distinguish fused from parental cells will make the selection of the polyploids strenuous, but not infeasible especially when a cell sorter can be used. The same is true for sexual fusion which allows to create autopolyploids that are only heterozygous at the mating type. The isogenic parental strains can be obtained by mating two closely related strains and performing a series of backcrossings till the offspring becomes nearly isogenic to one of the original parents (see, e.g., [94, 95]). Isogenicity should however be verified because the strains generated by Lin et al. [95] proved to be not entirely isogenic after 10 backcrosses, possibly the result of unintentional selection for mating efficiency [57].

As in the previous section, we want to emphasize that considering the potential instability of neopolyploids (especially colchipolyploids), it is key to monitor ploidy evolution in the weeks after their production.

6 Designing an Evolution Experiment with *Chlamydomonas*

Every experimental design is shaped by the hypothesis that it is supposed to test. The design of experiments aiming to quantify and qualify mutations, that is, mutation accumulation experiments (MAE) will be different from those of laboratory natural selection experiments (LNSE). Consequently, there is no standardized way to perform an evolution experiment. There are, however, several general choices that should be carefully considered when designing an experimental evolution study. We discuss them here specifically with the limitations and advantages of *Chlamydomonas* in mind.

Replication Replication and comparability are the main advantages of an evolutionary experiment that set it apart from the natural population-based and correlative methods that are otherwise used to study evolution. When brought in a new environment, adaptation can proceed along many different paths. Replication creates the unique opportunity to replay Gould's [96] "tape of life" allowing an assessment of the diversity of these adaptive solutions and the repeatability of evolution [97]. As in any other experiment, replication is also the key to statistical hypothesis testing; in this case, evolutionary hypothesis testing by comparing evolved replicates in the condition of interest with lines evolved in a control environment or even the preserved ancestral lines. More replicates will increase the statistical power, and although every replicate population increases the workload, it is strongly recommended to go for as many replicates as possible.

Growth medium *Chlamydomonas* laboratory cultures can be grown either in liquid medium or on agar plates (i.e., Petri dishes containing growth medium solidified with agar). Liquid growth medium allows the fastest growth, guarantees global interactions between the cells, offers the most practical way of subculturing, and facilitates counting and phenotyping of the cells, making it the medium of choice for most evolution experiments (especially LNSEs). On agar plates (typically 15 g agar L^{-1}), the cells are immobilized and give rise to colonies. This speeds up the detection of contamination and creates a pronounced spatial structure where interactions are localized and restricted to closely related cells. Furthermore, colony morphology can reveal features of individual genotypes. Even though the slower growth on agar is a serious disadvantage for evolution experiments, agar plates definitely do have their uses, for example, in MAE, where single cell bottlenecking is required and can even be indispensable for instance when studying adaptation in spatially heterogeneous environments.

Whether liquid or solidified, *Chlamydomonas* can grow on a wide variety of chemically defined media of which TAP [98], Bold's [99], and HSMT [100] are most commonly used. The species is vitamin independent, so there is no need for vitamin supplements, but since the laboratory strains cannot assimilate nitrate, a reduced nitrogen source is usually present [15]. The growth rate is highest under continuous light [101] and can be further increased by supplementing the medium with acetate (e.g., TAP) which is metabolized for mixo- or heterotrophic growth. Nevertheless, the higher growth rate comes at a cost as mixotrophic growth impedes synchronization even under a strict light-dark regime [61]. The optimal growth temperature for laboratory strains is 20–25 °C [15].

Culturing In liquid medium, cultures can be grown either continuously in a chemostat or by serial transfer in batch culture. Chemostats, that is, bioreactors with a continuous inflow of fresh medium and outflow of culture, provide the most efficient way to culture *Chlamydomonas*. The conditions are very stable and the growth rate is determined by the concentration of the limiting nutrient in the vessel and the rate of inflow (see [102]). But as chemostats are notoriously prone to contamination and the cost and complexity of the set up reduces the number of replicates, they are rarely used for *Chlamydomonas* experimental evolution.

Serial transferring is probably the simplest growth regime for *Chlamydomonas* lines (Fig. 4). A vessel with fresh medium is inoculated with cells, and after a few growth cycles, a fixed proportion of this culture is transferred to a new vessel with fresh medium. Models show that under such a regime there is strong selection for an increased growth rate and a reduced lag time. If the dilution

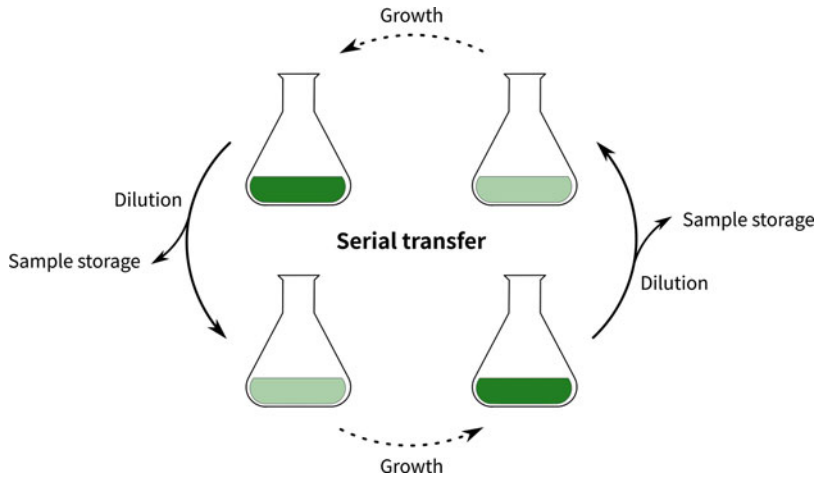

Fig. 4 Serial transfer in microbial experimental evolution

factor is large, selection will predominantly act on growth rate, whereas lag time will be affected more if the dilution factor is small [103]. If the cultures are transferred when the nutrients are limiting selection for starvation, survival might become important as well. Beside dilution factor and transfer time, population size is a third important parameter for serial transfer experiments. It is mainly determined by the amount of nutrients available or the size of the vessel when the growth medium is kept constant. Classical theory predicts higher rates of adaptation in larger populations, but population size is especially relevant to limit the effects of drift. Drift is mainly effective in small vessels and when bottlenecks are strong. Consequently, it is advised to use an effective population size of at least 5×10^4 cells [28]. The effective population size in asexual populations in batch culture is approximately the harmonic mean of the population size during the growth cycle and can be calculated as $N_e = N_{start} \times g$, where N_e is the effective population size, N_{start} is the number of cells in the inoculum, and g is the number of generations in between transfers [104]. Hubbarde and Wahl [105] calculated that a dilution factor of 5 maximizes the fixation probability of beneficial mutations and as such the rate of adaptation, but they did not take the potential effects of clonal interference into account.

Experimental lines can be grown in any laboratory vessel. Well plates are commonly used in *Chlamydomonas* experimental evolution, but they are prone to cross contamination when transferring the strains, so individually sealable vessels as Erlenmeyer flasks, culture flask, or test tubes should be considered as well. To reduce the risk on contamination, cultures should be handled in a sterile environment (i.e., a clean laminar flow) and filter tips should be used at all time.

Preservation The ability to preserve populations in a metabolically inactive state at different time points is highly advantageous in experimental evolution. Preserved strains can serve as a general backup, can be used for replay experiments, and are vital for direct comparison of ancestral with evolved lines (see below). *Chlamydomonas* strains can be kept on agar slants (typically 20 g L^{-1}) in dim light at ~18 °C. This method is very useful to keep strains for 2 to 12 months without refreshment; be that as it may, slants must be inspected regularly and refreshed when they start to bleach, turn olive, or the agar starts shrinking. Cultures on agar slants are not entirely metabolically inactive, and because the whole plating and recovery process could impose a selection pressure on or bottleneck the population, it is less well suited to preserve a heterogeneous evolved population. Cryopreservation is a better alternative for this purpose. The combined effects of a high recovery rate (>40%) when 5% methanol is used as cryoprotectant [106], and the fact that millions of cells can be preserved simultaneously in a single

cryo-tube minimizes the effects of drift and potential selection during the freeze-thaw cycle. Apart from storage of heterogenous cultures, cryopreservation is the method of choice for long-term storage (i.e., years). Good protocols for cryopreservation can be found in Crutchfield et al. [106] and in the methods section on the website of the *Chlamydomonas* resource center[3].

7 Quantifying Evolution

Evolution is defined as change in the heritable properties of a population over different generations. Quantification of such change is a central theme in evolutionary experiments. As both the phenotype and genotype are subject to change, evolution can be assessed at both levels.

7.1 Phenotype

Fitness is without doubt the most relevant phenotypical trait to monitor from the perspective of natural selection, even in MAE, where it is often of interest to measure the effects of new mutations on fitness (e.g., [107]). Yet, fitness is a complex trait, even for asexual single-cell organisms that reproduce by fission such as *Chlamydomonas*. Fitness does not depend uniquely on growth rate but also on the quality of the offspring produced. This is rather nicely illustrated by "prodigal son dynamics," that is, the fact that fast-living populations in an enriched environment have to return to ancestral growth rates because the fast growth compromises the quality of the cells [108]. Even so, growth parameters are still the most commonly used proxies for microbial fitness. Furthermore, fitness depends on the environment, which is often variable, consequently in experimental evolution the environment is often held constant (or at least assumed to be constant).

Growth assays The assessment of *Chlamydomonas* growth is relatively easy; it requires the transfer of a small number of cells to fresh medium containing enough resources to support substantial growth, followed by repeated measurement to quantify the increasing population size over time.

The resulting growth curves can be used to extract growth parameters such as yield at stationary phase, yield at the transfer interval, growth rate, and lag time. When studied separately, these parameters might give contradictory results, but a more

[3] https://www.chlamycollection.org/methods/

comprehensive estimate of fitness can be obtained by studying them together.

The number of individuals in the population can be measured either directly by cell counts or by using a proxy such as optical density (OD). For counting, traditional methods such as hemocytometer counts and plating dilution series are an option, but high-throughput-methods using coulter counters and (imaging) flow cytometry simultaneously provide additional information on the phenotype such as cell size and shape. As mentioned above, when using these methods for phenotyping, the use of synchronized cultures or gametes is a prerequisite to ensure that all cells are at the same stage of the cell cycle. Spectrophotometric evaluation of the optical density as a proxy for cell numbers is probably the fastest method to track growth over time especially when a plate reader is used. The relationship between optical density and cell number is not linear over the entire range of OD values and is influenced to some extent by cell size (and consequently by ploidy) and pigment content. By measuring at a wavelength with low absorption by chlorophyll a and b, such as 550 or 750 nm, linearity can be maintained for cell densities between 200,000 and 10 million cells mL^{-1} [109, 110]. Even though the lower concentration seems to approximate the detection threshold of many plate readers, one should be wary that measurements at low and high densities might be inaccurate, and it remains important to make separate calibration curves for every strain and growth condition combination especially if one wants to extract cell count information such as yield at the stationary phase.

Growth parameters are commonly extracted from growth curves by fitting a growth model. We found the Baranyi model [111], fitted with the Levenberg-Marquardt algorithm [112, 113], to provide the best results but depending on the strain and the conditions other models might fit the data better.

Relative fitness In experimental evolution, researchers are often more interested in change in fitness or differences in fitness rather than in fitness itself (e.g., in the context of ploidy level difference, an important result might be differences in fitness between haploid, diploid, and tetraploid strains). Normalization of the fitness measurements depends entirely on the hypotheses and the design but often involves a comparison of the ancestral and evolved lines – $(evolved fitness - ancestral fitness)/ancestral fitness$ – to compare adaptation between different lines [25].

Competition experiments Alternatively, relative fitness of the evolved compared to the ancestral lines can be quantified by growing both in competition starting from an equal number of cells. The competitive fitness is more inclusive than a comparison of, for example, the maximal growth rates of isogenic cultures and is

closest to the standard meaning of fitness in evolutionary theory [114]. Although widely applied in other microorganisms such as yeast and bacteria, this method has as far as we know never been used in a *Chlamydomonas* evolution experiment because of the technical limitation to distinguish between the two strains in mixed cultures. Indeed, the competing strains have to be marked (e.g., by any genetic marker followed by qPCR, by phenotypical markers such as color or even by resistance genes followed by selective plating), and the marking methods are technically quite difficult, time consuming, and might come at a fitness cost. Nevertheless, due to increasing efficiency of *Chlamydomonas* transformations and precision on genome editing (CRISPR), we expect to see competing fluorescently labeled or genetically tagged *Chlamydomonas* strains soon. Another drawback of competition assays is that the fitness difference between the competing strains should be limited. If one strain is substantially less fit than the other, measuring errors might cause a considerable error on the estimate of its abundance and fitness [114].

In addition to relative fitness, competition assays can be used to quantify evolution by revealing selective sweeps. To this purpose, the competing/marked strains should be identical except for the marker, and the assay is not performed to quantify the outcome of an evolutionary experiment but is the experiment itself. The replicate populations all contain an equal number of individuals of each marker type and evolve in competition. The rate of fixation or the frequency and the amplitude of the oscillations are consequently used to make inferences about the underlying evolutionary processes. Despite the technical differences related to the use of marked strains mentioned above, this method has been applied successfully in *Chlamydomonas* [32]. Within the context of whole-genome duplication, the first experimental evolution focusing on ploidy differences utilized an related method. In this experiment, Paquin and Adams [41] followed the mutation-based dynamics of a neutral marker in the asexual population to infer the number of adaptive mutations fixed, which they incorrectly synonymized with adaptation rate [44]. Later on, Paquin and Adams method was deemed unreliable because the observed fixation rates were too high given the population size [115]. Nevertheless, their work inspired others to use competitive evolution in their comparison of haploid, diploid, and tetraploid yeast (see, e.g., [47]).

Although the environment in most experimental evolution studies is held constant, this is not always the case, and for good reason in nature, environmental change is often gradual, and the dynamics of adaptation in gradually changing environments have shown to differ from those in which gradual change is applied (e.g., [32]). In those cases, competition or comparison of the evolved

and ancestral strains might have lost much of its original value, especially once the stress conditions become lethal to the ancestors. For these experiments and experiments on tolerance and resistance in general, the minimum inhibitory concentration might be a more valuable fitness proxy – examples of use of this proxy can be found in Weinreich et al., 2006 [116] and in the first experimental evolution ever conducted (at the end of the nineteenth century, the Reverend W.H. Dallinger built a bioreactor, inoculated it with flagellates, and increased the temperature over time from 15 °C to 70 °C).

Other phenotyping techniques Evolution is more than adaptation and fitness, and in the end, natural selection is only one of the major forces in evolution (next to recombination, drift, and mutation). Consequently, change in any phenotypical or genotypical (see next section) trait can be used to study evolution. However, one must be aware of the potential confounding effects of phenotypical plasticity, the evolution of which can be studied as well. Interesting phenotypic traits for *Chlamydomonas* are cell size, photosynthetic efficiency (measured, e.g., with a PAM fluorometer), respiration, pigment composition, nutrient composition, fatty acid composition, colony morphology, protein content, transcription, etc.

7.2 Genotype

Genetic changes can take many forms such as single nucleotide polymorphisms (SNPs), small and large insertions and deletions, structural variants (SVs) such as copy number variants (CNVs), variation caused by transposable element (TE) activity, inversions, translocations, and duplications. In most evolutionary experiments and population genomic studies, there is a strong focus on SNPs because these variants are relatively easy to call in next-generation sequencing data and there is a rich variety of detection techniques available. On the other hand, the adaptive role of other variants, such as CNVs, has been somewhat neglected, and there is evidence that these might be equally, or in some cases even better, fit to establish genetic differentiation and genotype-environment associations [117]. In the context of polyploidy, which is known to be linked to an increased TE-activity, large-scale deletions, and complex structural variation [118], SNPs alone might give a very incomplete picture of the genetic changes that shaped phenotypic evolution during experiments. As such, it is highly recommended to expand analyses to these other variants.

Sequencing technology One way to facilitate the detection of non-SNP variants is the use of long-read sequencing such as nanopore sequencing (e.g., by Oxford Nanopore Technologies, ONT), and single molecule real-time (SMRT) sequencing (e.g., by Pacific Biosciences, PacBio). These third-generation sequencing

technologies typically provide reads with lengths over 10 kb (up to 25 kb for SMRT and even Mbs for ONT) and can be extremely useful for the detection of complex structural variants. Error rates are rather high, consequently when accuracy is important, for example, for SNP calling, they should be combined with second-generation high-accuracy sequencing technologies such as Illumina. Long reads facilitate haplotype phasing, that is, separating the different genome copies present within the same individual (see [119] for an overview of phasing techniques) which is highly relevant in the context of polyploidy. Finally, third-generation sequencing has the potential to provide information about epigenetic base modifications [120–122].

Sequencing strategy Irrespective of which variants one wants to detect, two sequencing strategies can be applied. The first, classic, strategy consists of sequencing isogenic populations derived from single cells/colonies in each of the lines. This allows establishing the links between the different variants. When the sample size per evolutionary line is large, allele frequency estimates are reasonably accurate, and low-frequency variants and mutator genotypes can be easily detected. Alternatively, the evolved lines can be sequenced as a pool of individuals, that is, pool-seq. Pool-seq produces more accurately allele frequency estimates than single sample sequencing (at least when coverage is high enough). Additionally, this method reduces library prep costs substantially, as a single library suffices for each evolved line. Nevertheless, pool-seq does have some drawbacks. Because the frequency of a variant can take every value between 0 and 1, it is difficult to distinguish low-frequency variants from sequencing errors unless there is temporal replication (e.g., [123]). Consequently, under the single time-point scenario, a new variant should reach a certain abundance within the population before it can be called with certainty. As such, the method is probably most useful to detect changes in allele frequencies in populations with a high-standing genetic variation and less for the detection of new variants in originally isogenic populations. When comparing populations of different ploidy, this problem is exacerbated by the fact that the frequency of any variant decreases with ploidy, that is, if present in 10% of the population, you will get a frequency of 0.1, 0.05, 0.025 for haploids, diploids, and tetraploids, respectively. Accordingly, when a threshold is applied to remove the noise, you are bound to lose an unequal amount of real variants for samples of different ploidy. A related problem presents itself when a variant should have a certain absolute coverage to be detected. This latter problem can be easily remediated by standardizing coverage in a ploidy dependent way. To minimize loss of data, it is advisable to try to obtain a difference in coverage of the different samples at the sequencing level. Additionally, in the absence of temporal replication, pool-seq is not the most optimal tool to establish the links between mutations, especially when short reads are used.

8 Concluding Statement

During the past 6 years, we have experimented extensively with haploid and diploid *Chlamydomonas* strains, completing a first evolution experiment and starting a second one. Many of the recommendations given above come from the experience acquired during these experiments, sometimes by trial and error. We are convinced that *Chlamydomonas reinhardtii* is an excellent system to study WGD and subsequent evolution. We hope this chapter will inspire you to follow in our footsteps, and if so, when you need any additional advice or you encounter any problems, feel free to contact us.

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