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Studying Whole-Genome Duplication Using Experimental Evolution of *Spirodela polyrhiza*

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Abstract

In this chapter, we present the use of *Spirodela polyrhiza* in experiments designed to study the evolutionary impact of whole-genome duplication (WGD). We shortly introduce this duckweed species and explain why it is a suitable model for experimental evolution. Subsequently, we discuss the most relevant steps and methods in the design of a ploidy-related duckweed experiment. These steps include strain selection, ploidy determination,

different methods of making polyploid duckweeds, replication, culturing conditions, preservation, and the ways to quantify phenotypic and transcriptomic change.

1 Introduction

Ploidy shifts or whole-genome duplications (WGDs) are omnipresent, both within the tree of life and at all levels of biological organization [1]. With over 35% of all flowering plant species being polyploids, 15% of all spermatophyte speciation events coinciding with ploidy increase (for lycophytes and monilophytes, this amounts to even 31%) [2], and a growing number of documented paleopolyploidization events, WGD was and still is especially important for plant evolution. The exact mechanisms by which WGD fuels evolution are largely unknown, but it is widely believed that WGDs increase the adaptive potential, especially in stressful environments [3]. Most of the evidence for this hypothesis comes from observational studies relying either on the comparison of natural polyploids with the contemporary representative(s) of their assumed ancestral lineage(s) of lower ploidy or of diploids with artificial neopolyploids. Both comparisons have their flaws. The first comparison does not allow a separation of the effects of polyploidization from those of the subsequent evolution of the "ancestral" diploid(s) and the polyploid. The second comparison is limited by a restricted time frame that is generally too short to make inferences about evolution. This last problem is not imposed by the method itself but by the rather lengthy generation time of plants, and its solution lies in the use of organisms with a less constraining generation time such as the unicellular eukaryotes *Saccharomyces cerevisiae* (yeast) and *Chlamydomonas reinhardtii* (unicellular green alga) which have both been used to compare the evolution of ploidy variants (see also chapter by Bafort et al. in this issue [4]).

We here suggest the use of fast-growing duckweeds and more specifically *Spirodela polyrhiza* (from this point onward referred to as *Spirodela*), as an additional and a taxonomically more appropriate model system to study angiosperm polyploidization. Duckweeds or Lemnoideae is a clade of small aquatic macrophytes in the Araceae [5]. Their morphology is an example of extreme reduction and miniaturization. A plant typically consists of

the developmental hybrid between leaf and stem, called the frond [6], and, depending on the species, none, a single, or multiple root(s). Reproduction is predominantly vegetative, and with doubling times as low as 1.34 days [7], they are the fastest growing embryophytes. Lemnoideae went through two rounds of genome duplication during the Cretaceous [8], making them an ideal model system for ploidy research. Furthermore, their aquatic lifestyle, low space requirements (they are also the smallest among the higher plants), and high clonal growth rate made duckweeds a popular plant model system in the pre-molecular era when plant research was not yet dominated by *Arabidopsis*, and they seem to be regaining popularity in recent years. Consequently, the already considerable pool of methods, resources, and knowledge relevant to duckweed research is only growing, and there is a vibrant community centered around The International Steering Committee on Duckweed Research and Applications (ISCDRA, website: http://www.ruduckweed.org/).

With a frond size of up to 15 mm, *Spirodela polyrhiza* (Fig. 1) is the largest of all duckweed species, hence its common name greater/giant duckweed. *Spirodela polyrhiza* can be distinguished from its morphologically similar sister species *Spirodela intermedia*, by its capacity to form turions, manifested as small dark-brown/green-thick rootless fronds, the prophyllum, that is, a scalelike leaflet covering the root attachment point (Fig. 1b), being penetrated by only one or rarely two roots (in *S. intermedia*, two to five roots penetrate the prophyllum), and under some growth conditions by the presence of a red dot just above the node on the upper face of the fronds [9]. *Spirodela* has a nearly cosmopolitan distribution, occurring in temperate and tropical freshwater habitats around the world (with the notable exception of southern South America, some islands, and New Zealand) [10]. Its genome size is the smallest of all known duckweeds, that is, 138 Mb [8], making it an exemplary system for evolve and resequence experiments.

2 Strain Selection

Any species used to study the effects of WGD must come in strains of different ploidy. Ploidy determination is unfortunately not required when submitting a strain to a germplasm collection. Luckily, Urbanska-Worytkiewicz [11], Geber [12], Wang and coworkers [13], and Hoang and coworkers [14] have gone

through a great deal of work to determine the ploidy of over 180 cultivated *Spirodela* strains. One of these, the Puerto Rican strain 7110 (Landolt Duckweed Collection), proved to be a tetraploid [12]. A single tetraploid strain will not suffice for most experiments, but there are methods (described below) that allow the production of neo-tetraploids.

Diploid *Spirodela* has a limited genetic diversity with a clear geographic population structure consisting of an American, a European, an Indian and a Southeast Asian cluster [15]. On the other hand, differences in relative growth rate, plastic responses to temperature, salt tolerance, turion production, etc., [7, 16,17,18,19] reveal a rich phenotypic diversity both between and within these genetic clusters. Strains of all different ecotypes are readily available and can be obtained from several duckweed collections. The Landolt collection, founded by and named after Elias Landolt, the godfather of duckweed research, is the oldest and once was the most important collection harboring over 1000 strains collected from all over the world [20]. Today, the Rutgers Duckweed Stock Cooperative (RDSC) has taken over its role as preeminent collection, although there are many other collections [21]. Alternatively, strains can easily be isolated from the wild. Because of the similarity with *S. intermedia* (see above), the morphological identification should always be verified using molecular techniques such as barcoding [22, 23] or PCR-based methods such as amplified fragment length polymorphism (AFLP) fingerprinting [23], tubulin-based polymorphism (TBP) fingerprinting [24], polymorphic NB-ARC-related genes [25], or microsatellite arrays [26]. Axenicity of fronds isolated from nature can be achieved by submerging the plants for thirty seconds to three minutes in a 10–20% bleach solution with agitation and subsequently washing the fronds twice in demi-water to remove all traces of bleach. Usually, this procedure must be repeated several times. After recovery (up to two weeks after bleaching the fronds), the presence of any remaining bacterial contaminants can be checked microscopically or by growing the plants in medium containing 25 mM glucose.

3 Determining Ploidy

Ploidy level determination, whether of newly isolated strains, of newly synthesized potential polyploids, or of possibly diploidizing experimental strains, is one of the most important procedures in ploidy-related experiments.

Polyploidy in *Spirodela* causes a series of characteristic changes in morphology that can be used as a first indicator of ploidy change, including an increased frond (Fig. 2a) and stomatal guard cells size and a decrease in stomatal density (Fig. 2b). To facilitate the visibility of the stomatal guard cell, the fronds can be submerged in 70% ethanol solution, boiled for five minutes at 80 °C, washed for two minutes in fresh 70% ethanol solution, transferred to 10% bleach solution to remove any remaining pigmentation, and washed twice in demineralized water to remove all remaining ethanol and bleach. Because size and stomatal characteristics are rather plastic and depend on both strain and environment, these proxies should always be used with care and in combination with more reliable methods such as chromosome counts or flow cytometry.

Due to the rarity of flowering in *Spirodela*, chromosome counts are typically performed on metaphase spreads. Although any young tissue fixed in a 3:1 ethanol:acetic acid solution can be used [11], the efficiency is highest when using root tips treated with a mitotic inhibitor such as 2 µM of 8-hydroxyquinoline for 1 hour to cause metaphase arrest (for a full protocol, see, e.g., [14, 27]). Considering a diploid chromosome number of 40 and the size of these chromosomes, counting chromosomes can be difficult, especially when you are working with tetra- or octoploids.

Flow cytometry (Fig. 2c) offers a less cumbersome and higher-throughput alternative for ploidy determination. The nuclei can be extracted by taking a single *Spirodela* frond, washing it in water or fresh growth medium and chopping it for about a minute with a clean scalpel or razor blade in a Petri dish containing 500 µl ice-cold Galbraith's buffer [28] with 1% PVP brought to pH 7.0 with NaOH (filter sterilized using a 0.22 µm membrane filter). After chopping, slant the Petri dish, collect and mix all material, and leave inclined for another minute before mixing the material and transferring it over a 50 µm cell strainer to a centrifuge tube. Keep the extracted nuclei on ice. Add 10 µl of 0.2 mg ml^{-1} DAPI stock solution to 500 µl of extract, mix by vortexing, and run on the flow cytometer with appropriate channel. Keep the samples protected from light as DAPI is light sensitive, and fluorescence fades quickly when exposed to light. Analyze at least 5000 nuclei. Apart from DAPI, other stains such at propidium iodide or Sybr green I can also be used; the choice of strain

will depend on the lasers of the flow cytometer; DAPI has an excitation peak at 359 nm.

4 Making Polyploids

As mentioned above, the scarcity of known natural tetraploids for *Spirodela* invokes a need to create synthetical polyploids. Autopolyploidization of duckweeds is relatively easy and has been reported for *Landoltia* [29], *Lemna* (Tia-Lynn Ashman, personal communication), and *Spirodela* (unpublished work within our group). Currently, we have autotetraploids from strains belonging to each of the geographical subpopulations described by Xu et al. [15], available to all researchers upon request. Autopolyploidization can be achieved by treating the strains with colchicine, a natural alkaloid found in the autumn crocus (*Colchicum autumnale*). The chemical has an affinity for and depolymerizing effect on the beta tubulin units of the microtubules, which can cause metaphase cell cycle arrest. The best results were obtained (by our group) when fronds were incubated in Hoagland medium [30] supplemented with 0.7% colchicine and 0.5% DMSO (to increase penetration in the cell) under a 16-8 light-dark light regime for 24 h at 40–45 µmol m^{-2} s^{-1} PPFD and 24 °C. Following colchicine treatment, fronds need to be washed three times in ddH_2O to remove the colchicine before transferring them to regular medium for a slow recovery. After 3–5 weeks, the ploidy can be determined by using the methodologies described above. The success rate is rather low, so it is important to start with a population of minimum 50 fronds.

As far as we know, allopolyploid duckweeds, in which both genome copies differ substantially from each other, have never been reported. Braglia and coworkers [24] described natural hybrids between *Lemna minor* and *Lemna turionifera*, but they did not determine their karyotype, so it remains unclear whether polyploidization was involved. The difficulties to induce flowering in *Spirodela* makes natural hybridization followed by allopolyploidization unlikely, nevertheless, it should in theory be possible to create synthetic allopolyploids using somatic cell fusion. The successful creation of such a somatic hybrid depends on protoplast generation, protoplast fusion, and regeneration. Regeneration from callus is possible [31], and although protoplasts can be generated from *Spirodela* root material, the protocols can

be (and are being) improved in terms of efficiency (personal communication with Anthony Bishopp, Alex Ware, and Carolin Seyfferth). The remaining step, protoplast fusion, still must be tested on *Spirodela*, but symmetrical cell fusion using electrical fields has been tried already on the related Aracean genera *Spathiphyllum* and *Anthurium* [32]. Although no hybrids were recovered and growth stopped after the production of microcalli, this was an attempted intergeneric hybridization, and we believe interspecific crosses could be brought to a more satisfactory end.

5 Designing *Spirodela* Evolution Experiments

Experimental evolution is the study of replicate populations under defined and reproducible conditions over the course of many generations [33] and is one of the most potent methods to study evolution [34]. Although natural or anthropological-driven events such as introduction events and environmental pollution can be secondarily analyzed and considered as evolutionary experiments [33], here, we restrict ourselves to a discussion of more controlled laboratory evolution experiment. Such experiments can take many forms, and the design should be neatly adjusted to the specific question(s) asked. Nevertheless, some general types of laboratory evolution experiments can be distinguished. We will shortly discuss the four that are most used before diving into the more practical and general aspects of these experiments.

5.1 General Designs

Laboratory natural selection experiments (LNS) are probably the most typical evolutionary experiments. Replicate populations are brought into a novel-controlled environment, imposing them to a new selection pressure. A random sample of the population, naturally enriched for fitter individuals, is used to initiate the next generation, that is, serial transfer. Because this is a random sample that does not necessarily include the fittest individuals as in culling and artificial selection experiments, population sizes of natural selection experiments should be larger to obtain the same rate of change. By controlling the population size, it is possible to adjust the relative contribution of drift and selection [35].

In laboratory culling experiments, the experimental populations are exposed for a short period of time to a lethal environmental stress. When only the most resistant individuals remain, these are recovered and brought into a benign environment where they are maintained to rear the next generation which will, in turn, be exposed to the same stressor and so on. In these experiments, any trait that increases survival in the lethal environment contributes to the fitness. Phenotypical heterogeneity, i.e., a form of variability is supposed to be favored under such a regime.

Artificial truncating selection experiments use the same principles that have been used for centuries by plant and animal breeders. All individuals within the population are scored for a particular (phenotypic) trait, and the obtained ranking is subsequently used to select the fittest fraction of individuals (commonly a fixed percentage of the population size) to start the next generation. Often the highest or lowest ranking individuals are selected (directional selection), but the method also allows for disruptive and stabilizing selection. By manually selecting the "fittest" individuals based on a specific trait, the experimenter creates a direct link between fitness and this trait. Although this holds to some extent for LNS, there the strong selection pressure for increased growth rate imposed by serial transfer can be counteracted if high growth rates are disadvantageous (see, e.g., Prodigal Son dynamics [36]). For this reason, some evolutionary biologists [37] do not consider selective breeding as experimental evolution.

Mutation accumulation experiments are a completely different type of evolution experiments, by applying strong population bottlenecks, for example, a single individual per generation, genetic drift is maximized and selection reduced, which allows the accumulation of beneficial, neutral, and nonlethal deleterious mutations. The method is useful to estimate the mutation rate, and when combined with phenotyping, it can be informative on the fitness effects of those mutations.

5.2 Replication

Much of the strength of experimental evolution lies in replication. When a population faces a new environmental condition, adaptation can proceed

along different paths. Depending on the path taken, the evolutionary outcome might be quite different. Replication allows an assessment of the variation in these outcomes and the paths taken to get there. As such, they might offer insights into the repeatability of evolution and can increase the power of statistical conclusions.

5.3 Growth Conditions

Duckweeds can be grown on a rich variety of growth media, but Hoagland solution [9, 30], Steinberg medium [38], N-medium [39], and Schenk-Hildebrand medium [40], or modifications thereof are most used. A more extensive overview and recipes can be found in Appenroth [41]. When fast mixotrophic growth is needed or to detect contamination, the medium can be supplemented with glucose (50 mM) or sucrose (25 mM) [41].

The culturing vessel used will depend entirely on the experimental design, especially on the population size and whether the exact number of generations must be known. Because duckweed grows vegetatively and fronds can survive for several weeks, it becomes hard to express evolution in terms of the number of generations in large populations under serial transfer. If one has to keep track of the number of generations, for example, for mutation accumulation experiments, fronds can be kept separated and individually labeled. Well plates are an ideal vessel for this purpose. When small populations are needed, for example, for phenotyping, Erlenmeyer flask, beakers, or small plastic boxes can be used. For long-term evolution experiments that require a large population to reduce the waiting time in between beneficial mutations, large open systems such as open boxes, aquariums, cattle tanks, or even ponds are more suitable. When using smaller vessels, a transparent bottom will allow bottom illumination which facilitates phenotyping using pictures taken from above, whereas for medium-scale open systems, black or dark boxes offer the best solution as they limit the exposure of the medium to light and as such suppress the growth of algae.

Optimal growing conditions might vary depending on the strain, but in general Spirodela is grown under a 16:8 light dark regime, temperatures of 23–25 °C, and 30–300 µmol m^{-2} s^{-1} PPFD. Appenroth [42] reported an increasing growth

rate between 30 and 90 µmol m^{-2} s^{-1} PPFD, followed by a plateau and a decrease in growth rate from 300 µmol m^{-2} s^{-1} PPFD onward. We grow our cultures at 40–45 µmol m^{-2} s^{-1} PPFD. Open outdoor systems are commonly covered by nets to reduce direct radiation and to create some shading (e.g., [43, 44]). Within such systems, it is possible to make smaller enclosures to quantify growth parameters.

Irrespective of the conditions, when duckweeds are transferred to a new environment, they will need some time to adjust (phenotypical plasticity). As such, it is important to acclimatize your cultures sufficiently long to the new environment before quantifying growth; according to the literature, it might take 4 weeks (even 6 when coming from agar) to get a constant growth rate when fronds are transferred to a new environment [7, 9]. Landolt sees this as a result of the buds being formed long before they become visible.

5.4 Preservation

During evolution experiments, it is fundamental that ancestral strains are preserved, for example, for competition assays. Currently there are two ways to achieve this. A first method is growing the plants under a low-maintenance slow growth regime. Plants can be kept in liquid medium under low light and lower temperature. Although duckweeds can be embedded in 0.8–0.9% agar, *Spirodela* stocks are best kept in liquid medium as the plant has the tendency to grow vertically on agar which will result in overtopping and even death, when the top layer loses its connection to the agar [21]. The ISCDRA recommends keeping duckweed stock cultures at 15 °C [45], but for some (tropical) strains, higher temperatures such as 17 °C might be better [21]. A second method is preservation as turions. These starch-rich overwintering propagules can be stored safely in the dark at 4 °C for years without requiring any maintenance. Although turion-based preservation is still in its infancy, the RDSC has developed a method for turion induction that seems to work [45]. A third method, cryopreservation of duckweed fronds, is still in its infancy but recently progress has been made at Kyoto U and IPK Gatersleben (Shogo Ito and Manuela Nagel, personal communication).

6 Quantifying Phenotypic Change

6.1 Fitness

6.1.1 Growth Rate

Fitness can be assessed in different ways. A first proxy is population growth rate. Typically, exponential growth is assumed and as such growth is quantified as the exponential growth rate or relative growth rate (RGR), expressed as RGR = (lnP1 - lnP0)/(t1 - t0), where P1 and P0 represent the population size and t1 and t0 the corresponding points in time. Often, population size is measured only twice, for example, 7 [7] or 10 [46] days apart. Landolt [9] however recommended the use of more measurements (at least three), followed by a linear regression. Although this method is more labor intensive, it will provide a more accurate estimate of growth rate and allows the detection of deviations from the assumed exponential growth.

Population size can be expressed in terms of fresh weight, dry weight, coverage (i.e., surface area), or frond number. When frond number is used, the growth rate comes closest to the rate of clonal reproduction that is often used as a proxy of microbial fitness. Nevertheless, it should be noted that this measure fails to account for changes in area and thickness of the fronds, unless these are measured separately. Additionally, counting fronds is labor intensive, especially when done manually. However, thanks to advances in image analysis software and machine learning algorithms, automated counts have become quite reliable. Such software works best if the pictures are taken from above with a light source put underneath the culture. Area measurements, dry and fresh weight, do not necessarily correspond well to the number of fronds, as the average frond size can change over time. Nevertheless, area is particularly relevant if competition for space is important. Under such a scenario, the strain that covers a large area fastest could be considered fittest, irrespective whether the higher coverage is obtained by a few large fronds or by many small ones with a high rate of reproduction. Fresh weight measurements are somewhat more inclusive as they not only give an idea of area but also of thickness and starch content. That said, fresh weight is relatively imprecise as the measurement might depend on the condition under which the samples are taken and prepared. Dry weight is more reliable as the samples are dried till the weight stabilizes and all water is gone. Nevertheless, fresh weight measurements can be done faster, and the same population can

be used for the two time points, whereas extra replicates or representative subsamples are necessary for dry weight estimation, which destroys the material. As all these different measurements reflect different aspects of growth and fitness, a combination of different measures gives the most inclusive results. Irrespective of the method used, there might be considerable variation between replicate growth rate estimates. This variation is mainly caused by differences in the starting material and can be reduced by starting with a larger population of 15–20 fronds [10].

6.1.2 Competition Assays

Competition assays provide a second way to assess fitness. When talking about competition, we must distinguish between exploitative and interference competition [47], both forms of competition can be inter- and intraspecific [48]. Exploitative competition is competition for a limited resource. For duckweeds and floating aquatic macrophytes, in general, these resources can be space, light, and essential nutrients [47, 49]. The relative importance of each of these resources is however unknown. Some argue that space is less important because growth continues when the entire surface is covered (i.e., overlapping), [43] but others are convinced that light is less important [50]. Interference competition is competition in which at least one of the competitors inhibit the growth of the other (e.g., allelopathy by using a chemical) or inhibits its competitors' access to one of the resources directly (e.g., by overtopping). Although interspecific allelopathy is common among floating aquatic macrophytes [47] and the negative allelopathic potential of some duckweed species has been established [51, 52], negative allelopathic effects of *Spirodela* seem to be mainly intraspecific [52]. Overtopping on the other hand has been reported as an important factor in interspecific duckweed competition assays including *Spirodela* [52]. Because competition has so many aspects, competition assays can be designed differently according to the aspects of competition one wants to study. In the most general competition assay, the evolved strains are competed, depending on the questions asked, against each other, their ancestor, or control lines evolving under standard benign conditions, and the strain that can outcompete or obtain the highest population sizes is considered as fittest. These assays are only an option if it is possible to distinguish between the competing lines. Manually labeling the

fronds, for example, with permanent marker and monitoring the cultures regularly to label the new daughter fronds, is a simple but time-consuming option that can be applied without too much difficulties as long as population densities remain low. When densities become higher and overtopping begins, special care should be taken not to disturb the fronds. Alternatively, if the competing lines are not isogenic, genetic markers such as microsatellites, for example, [26, 43, 53], inter-simple sequence repeats [54], amplified fragment length polymorphisms (AFLP), for example, [23], tubulin-based polymorphisms (TBP) fingerprinting [24], and polymorphic NB-ARC-related genes [25] can be used to distinguish the fronds at the end of the assay. When competing isogenic lines of different ploidy, bulk flow cytometry can be considered. A calibration curve using mixtures of different tetraploids and diploids can be used to account for endopolyploidy. Multiple large samples, that is, minimum 50 plants, should be used to obtain an accurate estimate of the proportion of each ploidy variant in the population. If only competition for nutrients is important, the cultures can also be grown in half-open enclosures that allow free movement of nutrients and metabolites but do not allow duckweed to move (see, e.g., [46, 55]). This setup can equally well be applied for the study of allelopathy-related questions, but it will be impossible to distinguished allelopathic effects from these of exploitative competition if some resource is limited. Alternatively, plant extracts of the competitor can be added directly to the medium, or populations can be grown in medium that was previously used to grow the potential competitor, for example, [52]. The confounding effects of exploitative competition can be removed by supplementing the nutrient levels in the used medium till they reach the levels of the control medium, for example, [56].

6.1.3 Stress Resistance

A third method consists of measuring the stress resistance directly through a tolerance assay. By exposing the evolving populations to a gradient of concentration of a stressor, it is possible to determine the half-maximal effective concentration (EC50) and to use these as a proxy of fitness. This method is particularly interesting if the stress level is increased over time and reaches levels where growth of the control population is entirely inhibited, making competition assays redundant. Alternatively, other indicators of stress

can be used to detect increased stress resistance. Starch content, for example, is known to increase in duckweeds under some stressful conditions such as phosphate depleted, saline (NaCl), and heavy metal (Cd, Ni)-enriched environments [17, 57, 58] and can be easily quantified qualitatively by cleaning the fronds with 70% ethanol followed by Lugol staining [59]. Alternatively, the starch content can also be quantified colorimetrically using the method of Magel [57, 60], conversion to glucose followed by glucose quantification using an enzymatic assay [61, 62] or HPLC [63]. Other indicators of stress that can be used are photosynthetic parameters as measured by PAM fluorometry (see below) and root regrowth [64].

6.2 General Morphology

In addition to fitness, there are many other phenotypical traits that can be monitored during experimental evolution. Changes in general morphology and anatomy can be easily detected by eye, but due to the very simple morphology of *Spirodela*, the number of traits is rather limited. Frond size (area), length, width, length/width ratio, fronds per colony, amount of necrotic tissue, and general health based on false-color imprints [65] are commonly used and can be easily measured on photographs. Frond thickness can be quantified using a thickness gauge but is regularly substituted by specific leaf mass (SLM), that is, the ratio of dry weight to area of leaf/frond. SLM is the product of leaf density and thickness [66]. Because root length varies with frond and root age, it is common practice to cut the roots and measure the length of new roots within a fixed amount of time [64].

6.3 Pigments and Photosynthetic Parameters

Pigment content can be quantified colorimetrically, for example, using the method of Lichtenthaler [67] for chlorophyll a, chlorophyll b, and carotenoids and the method of Mancinelli [68] for anthocyanins. But for more specific results, a combination of high/ultra-performance liquid chromatography (HPLC/UPLC) and mass spectrometry (MS) can be used. Pulse-amplitude modulation (PAM) fluorometry is commonly used to assess parameters related to chlorophyll fluorescence. Particularly interesting parameters are the maximum quantum yield of photosystem II, that is, the Fv/Fm (measured after 30 minutes dark adaptation), non-photochemical fluorescence quenching

(NPQ), that is, (Fm-Fm')/Fm', and the effective quantum yield of photosystem II or YII of illuminated samples, that is, the proportion of absorbed light that is used in photo system II photochemistry [69, 70].

7 Quantifying Transcriptomic Change

Although variable in time and extent, polyploidization can induce extensive transcriptomic changes [71]. The magnitude of change depends at least partially on the type of polyploidization. The effects of allopolyploidy are in general more pronounced than those of autopolyploidy, because the latter only involves genome doubling whereas the former includes the merging of two genomes with different regulatory mechanisms as well [72]. Surprisingly, the duplication of the genetic material is not necessarily translated into a doubling of transcription; instead the few studies in which transcriptome size was studied seem to suggest that the response can be anywhere between full dosage compensation (i.e., the tetraploid has a similar transcriptome size as its parents) and 1:1 dosage effects (i.e., transcriptome size is proportional to genome size) and that the effects are not uniform but may differ across genes [73].

Expression profiles are mainly studied using RNA-sequencing (RNA-Seq). RNA-Seq has several advantages over older transcriptomic technologies [74], and for polyploidy research in particular, this technology should be preferred for two reasons. First, RNA-Seq reveals sequence differences (e.g., SNPs), allowing identification of homoeologs (especially interesting for allopolyploids). Second, it can detect transcripts with a large range of expression levels, which is important when major expression level changes are expected, for instance, after genome merging. In the past decade, RNA-Seq has relied almost exclusively on Illumina high-throughput deep-sequencing technology [75]. Nevertheless, other (the so-called third-generation) sequencing technologies such as nanopore sequencing (e.g., by Oxford Nanopore Technologies, ONT) and single molecule real-time (SMRT) sequencing (e.g., by Pacific Biosciences, pacbio) are gaining popularity [75], and the length of these reads (complete RNA molecules converted to cDNA) might, in the near future when throughput increases and error rate decreases, become very attractive to those interested in homoeolog (duplicates created in a WGD event) discrimination. Extensive

reviews on RNA-Seq technology, RNA-Seq applications, and differential expression analysis can be found elsewhere [74,75,76,77].

Although most RNA-seq experiments focus exclusively on differential expression analysis, transcriptome size should not be ignored, as it will likely differ between cells of different ploidy. Traditionally, gene expression is depth-normalized, for example, the reads per million (RPM) value. This "traditional" normalization method does not provide any information about the transcriptome size or the relative transcriptome size, and when comparing two samples, no conclusions can be drawn about expression per cell or genome. Failure to account for differences in transcriptome size is often problematic in polyploidy research, for example, potential differences in parental transcriptome size might alter the outcome of expression bias studies. Additionally, dosage response studies are needed to shed light on the effects of polyploidy on gene expression [78, 79]. Consequently, the quantification of transcriptome size or at least the relative transcriptome size of the compared organisms should always be considered for ploidy related experiments and is even a necessity when dosage response and expression bias are subjects of interest.

When the number of cells used for RNA extraction is known (e.g., single-celled organisms [4]), gene expression can be normalized per cell by adding heterologous RNA (e.g., External RNA Controls Consortium RNA) proportional to the cell number and looking at the expression of the gene of interest relative to the proportion of exogenous RNA. If the RNA is extracted from an unknown number of cells, which will be the case for *Spirodela*, the (relative) transcriptome size must be quantified. J. E. Coate and J. J. Doyle [80, 81] pointed out that the relative transcriptome size, that is, the tetraploid to diploid ratio (TDR) of transcriptome size per cell can be estimated by dividing the TDR of the expression of a specific gene per cell by the TDR of the expression of that gene relative to the transcriptome size. Whereas the denominator can be easily derived from regular RNA-seq, the numerator requires a more creative solution. First, the relative expression per genome is calculated using RNA-genomic DNA (gDNA) coextraction, followed by reverse transcription from RNA to cDNA and a qRT-PCR to quantify the RNA/gDNA ratio. Subsequently, the ploidy information is used to translate this ratio to the

relative expression per cell. To increase the accuracy of the estimated relative transcriptome size, it should be calculated using several transcriptional markers all using the geometric mean of the amplification efficiency of at least three genomic markers.

If you consider such a normalizations, we highly recommend you to read Coates' excellent in-depth discussion of this topic elsewhere in this issue [82].

8 Quantifying Genetic Change

The quantification of genetic change in the context of experimental evolution and, in particular, evolve and resequence experiments has been discussed in detail elsewhere in this issue [4].

9 Concluding Statement

Although *Spirodela* and duckweeds have been used as a model system for many decades, the use of duckweed for evolution experiments is relatively novel. During the past 2 years, we have experimented extensively with diploid and tetraploid duckweeds, and many of the recommendations given above come from our own experience acquired during these experiments, sometimes by trial and error. During these experiments, we became convinced of the suitability of *Spirodela* to study WGD and subsequent evolution. We hope this chapter inspired the reader to follow in our footsteps and are always willing to provide additional advice on the use of duckweeds, in particular, *Spirodela*, in evolution experiments.

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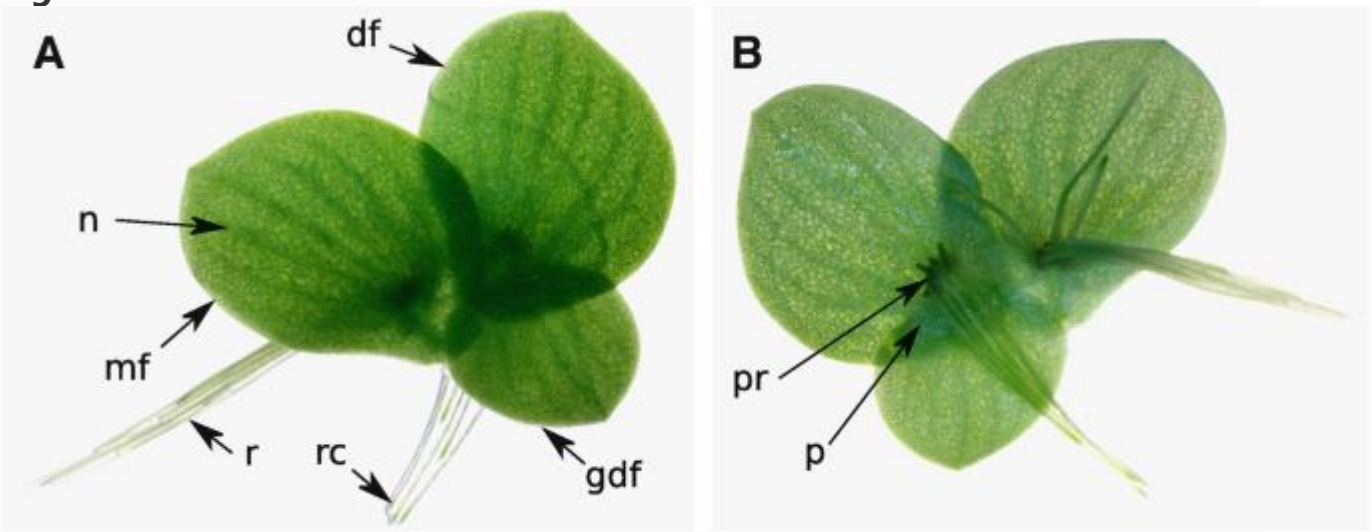
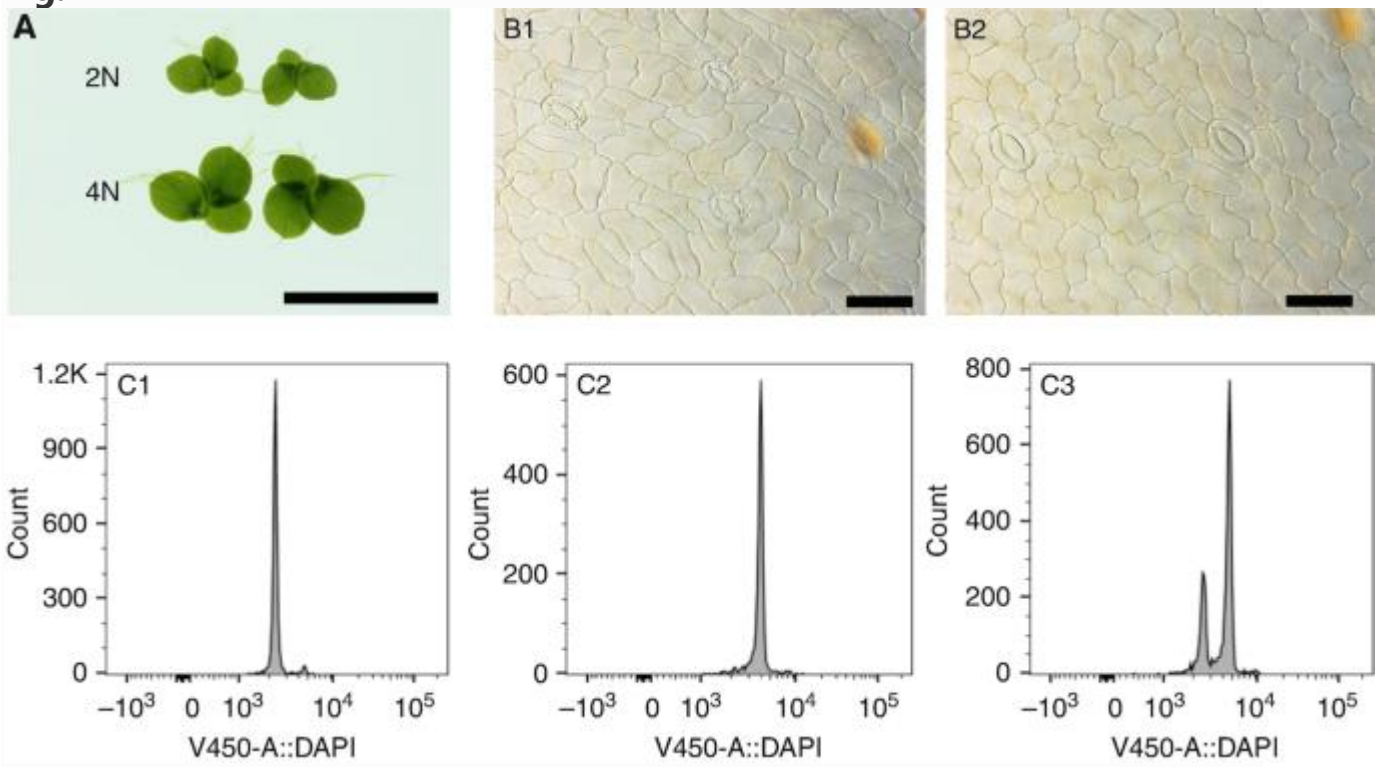
Fig. 1



Morphology of the dorsal (**a**) and ventral (**b**) side of a *Spirodela polyrhiza* plant. Mf: mother frond; df: daughter frond; gdf: granddaughter frond; n: nerve; p: pouch; pr: prophyllum; r: root; rc: rootcap

Fig. 2



Differences between diploid (2N) and tetraploid (4N) plants: (**a**) general morphology (scale bar 20 mm); (**b**) stomatal size and density of diploid (B1) and tetraploid (B2) plants (scale bar 40 μm); (**c**) flow cytometry of diploid (C1), tetraploid (C2), and mixed (C3) samples