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Alternating nuclear DNA content in chrysophytes provides evidence of their isomorphic haploid-diploid life cycle

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A B S T R A C T

Across eukaryotic organisms there is a great diversity of life cycles. This particularly applies to unicellular eukaryotes (protists), where the life cycles are still largely unexplored, although this knowledge is key to understanding their biology.

To detect the often inconspicuous transitions among life cycle stages, we focused at shifts in ploidy levels within strains of unicellular chrysophyte alga. Representatives of three genera (*Chrysosphaerella*, *Ochromonas,* and *Synura*) were analysed for nuclear DNA contents using a propidium iodide flow cytometry. Selected strains exhibiting ploidy level variation were also surveyed for DNA base composition (GC content) and cell size. Additionally, we tracked ploidy level changes in seven strains under long-term cultivation.

An alternation of two ploidy levels was revealed in the life cycle of chrysophytes with both life cycle stages capable of mitotic growth and long-term survival in cultivation. With the exception of a small increase in cell size with higher ploidy, both life cycle stages shared the same phenotype and also had highly similar genomic GC content. Further, we detected three ploidy levels in two *Synura* species (*S. glabra*, *S. heteropora*), where the highest ploidy (putatively 4x) most likely resulted from a polyploidization event.

Consequently, chrysophytes have a haploid-diploid life cycle with isomorphic life cycle stages. As far as we know, this is the first report of such life cycle strategy in unicellular algae. Life cycle stages and life stage transitions seem to be synchronized among all cells coexisting within a culture, possibly due to chemical signals. Particular life stages may be more successful under certain environmental conditions, for our studied strains the diploid stage prevailed in cultivation.

1. Introduction

Across eukaryotic organisms there is a considerable diversity of life cycles. Individuals at particular life cycle stages may differ, for example, by their overall morphology, environmental requirements, or by the number of chromosome sets in cell nuclei (ploidy level). Many organisms alternate between two stages, a haploid phase with one set of chromosomes reduced by meiosis and a duplicated diploid phase following the fusion of gametes [1,2]. Depending on whether the both stages are more-or-less equally represented in the life cycle or one of them largely predominates, various life cycles can be recognized. In a diploid life cycle, organisms switch between a short haploid phase (usually restricted to unicellular gametes) and the prevailing diploid phase, only which is capable of mitotic growth. Such a life cycle occurs among diatoms, raphidophytes or budding yeasts; however, it is best known from animals, including humans [3–6]. A haploid life cycle is characterized by mitosis restricted to the haploid phase, which also lasts for most of the organism's lifespan, as the only diploid stage is a unicellular zygote. The haploid life cycle evolved in stoneworts (charophytes) and in some other green algae [7]. However, probably the most widespread among organisms is a haploid-diploid life cycle. Here, both the haploid and diploid stages are capable of mitotic growth. This life strategy dominates in land plants, red and brown algae, basidiomycete fungi, but also occurs in many groups of green algae and various groups of unicellular algae [4,7–10]. A peculiar form of this life cycle evolved in some green and red algae, where the haploid and diploid phases are morphologically indistinguishable [11,12]. This isomorphic haploid-diploid life cycle can be found, for example, in sea lettuce (*Ulva lactuca*; [11]).

Knowledge of the life cycle and identification of particular life cycle

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stages are key not only to understand the basic biology of studied organisms, but also to correctly assess their genome size (1C vs. 2C value), and hence to design an optimal sequencing strategy, and properly interpret population genetic or genomic data [13–15]. In algae, understanding the life cycles is also essential to predict formation of blooms and toxins production [4]. However, the knowledge of algal life cycles remains still largely fragmented, particularly for most unicellular algae. This could be attributed to their microscopic size and a frequent lack of pronounced morphological features, which makes life cycle stage transitions harder to detect. Here, we attempt to overcome the problem by measuring nuclear DNA contents and looking for shifts in a ploidy level that should be associated with the life cycle transitions.

To broaden our knowledge of algal life cycles, we chose chrysophytes as a model group. The chrysophytes, also known as golden-brown algae, are single-celled or colonial flagellates, which occur primarily in freshwater phytoplankton and their blooms can cause an unpleasant fishy odour in drinking water reservoirs [16]. In some taxa (e.g. among the representatives of the genera *Synura* and *Chrysosphaerella*), the cells are covered by species-specific silica scales [17]. However, not much is known about the chrysophyte life cycle. Undifferentiated cells may serve as gametes [18]. Fusion of the gametes was observed in several cases, specifically the apical fusion in *Kephyrion*, *Stenocalyx*, *Chrysolykos* and *Dinobryon* or the posterior fusion in *Synura* and *Mallomonas* [18,19]. The fusion of gametes is followed by cyst formation [17]. Some colonial species even produce separate male and female colonies [20]. According to Sandgren and Flanagin [21], the genus *Synura* is heterothallic and its sexuality might be induced at high cell densities. In recent years, the chrysophytes have drawn attention due to their remarkable DNA content diversity, ranging from 0.09 to 24.85 pg (0.09 to 24.31 Gbp), accompanied by numerous cases of major intraspecific variability [22–24]. This variation was either attributed to polyploidization (i.e., whole-genome doubling) or its source remained unresolved [23,24].

The present study was stimulated by our repeated detection of intraspecific DNA content variation arising in cultures of some of our investigated taxa that opened the question whether this could be attributed to unprecedented rates of certain evolutionary processes (e.g. polyploidization, aneuploidization, proliferation of transposable elements [25]) or whether it constitutes an inherent part of organisms' life cycles. By employing flow cytometry on selected, DNA content variable taxa we are asking the following specific questions: 1) What are the patterns of DNA content variation among and within strains; and do these correspond to ploidy level shifts (i.e., two-fold DNA content differences)? 2) Of what character are the temporal changes in the DNA content of strains over time in cultivation? 3) Is intraspecific / intrastrain DNA content variation linked with differences in genomic base composition (i.e. GC content; no differences expected under the scenario of whole genome duplication)? 4) Are there any apparent phenotypic differences between intraspecific strains with different DNA contents?

2. Materials and methods

2.1. Origin and cultivation of the investigated strains

For this study, we selected chrysophyte taxa where intraspecific DNA content variation was detected during our previous unpublished work. Altogether 61 chrysophyte strains were obtained from 49 various freshwater localities across the Northern hemisphere, comprising 59 isolates of the genus *Synura*, one isolate of *Ochromonas tuberculata* and one isolate of *Chrysosphaerella brevispina*. The sampling details are listed in Supplementary data Table S1. To establish new cultures, water samples were taken using a 25 μm mesh plankton net and single cells or colonies were captured by micro-pipetting and transferred into separate culture wells filled either with MES buffered DY-IV (in case of *S. sphagnicola* and *Ochromonas tuberculata* [26]) or with WC medium [27]. The culture collection was supplemented with seven previously established cultures [23,28–30]. All cultures were maintained at 17 °C (cooling box Pol-Eko Aparatura Sp.J., model ST 1, Wodzisław Śląski, Poland) with a 24-h light mode under illumination of 30 μmol m^{-2} s^{-1} (TLD 18 W/33 fluorescent lamps, Philips, Amsterdam, Netherlands). The generation time of *Synura* cells under these cultivation conditions can be approximated as 2 days (based on Kim et al. [31]). Subsequently, the strains were transferred into Erlenmeyer flasks filled with 30 mL of growth medium and kept for longer cultivation with re-inoculations into a fresh medium every three months.

2.2. Phylogenetic analyses

To genetically identify *Synura* strains, the internal transcribed spacer of nuclear ribosomal DNA (ITS1, 5.8S and ITS2 rDNA; nu ITS rDNA) of individual isolates was sequenced. For this purpose, genomic DNA was extracted from a centrifuged pellet of cells by InstaGene Matrix (Bio-Rad, Hercules, CA, USA), and the resulting supernatant directly used as a PCR template. Amplifications were performed using the universal primer ITS4 [32] and a genus-specific primer Kn1.1 [33]. PCRs were carried out in a total volume of 20 μL with a PCR mix containing 0.2 μL of MyTaqHS DNA polymerase (Bioline, Memphis, TN, USA), 4 μL of MyTaqHS buffer (Bioline), 0.4 μL of each primer, 14 μL of double distilled water and 1 μL of template DNA (not quantified). Amplifications were performed in Eppendorf Mastercycler ep Gradient 5341 (Eppendorf GmbH, Hamburg, Germany) using the following program: 1 min of denaturation at 95 °C; followed by 35 cycles of denaturation at 95 °C (15 s), annealing at 52 °C (30 s) and elongation at 72 °C (40 s), concluded with a final extension at 72 °C (7 min) and held at 10 °C. The PCR products were sized on a 1% agarose gel and then purified using AMPure XP magnetic beads (Agencourt, Beckman Coulter, Brea, CA, USA). The purified DNA templates were sequenced using the Sanger sequencing method at Macrogen, Inc. (Amsterdam, Netherlands, https://dna. macrogen.com). Finally, the obtained sequences were identified using BLAST in the National Center for Biotechnology Information (NCBI) Search database and our own ITS database built up during previous studies [23,29,30,34,35].

The phylogenetic tree was inferred by the maximum likelihood (ML) analysis using RAxML 8.1.20 [36], applying the GTR + Γ evolutionary model. Bootstrap analysis was performed with the rapid bootstrapping procedure, using 100 pseudoreplicates. Bayesian posterior probabilities were computed by MrBayes 3.2.6 [37]. Two parallel Monte Carlo Markov chains runs were carried out for 3 million generations each with one cold and three heated chains. Trees and parameters were sampled every 100th generation. Convergence of the two runs was assessed during the run by calculating the average standard deviation of split frequencies. The "burn-in" was specified at the value 1000 using the "sump" command. All analyses were run at the Cyberinfrastructure for Phylogenetic Research (CIPRES) Portal (http://www.phylo.org/sub_sections/portal; [38]).

2.3. DNA content estimation and ploidy level assignment

To estimate nuclear DNA contents of the obtained strains, we employed propidium iodide flow cytometry (PI FCM). Approximately two weeks before the planned FCM analyses, cultures were inoculated into fresh medium. For sample preparation, 1 mL of well-grown culture was centrifuged (5500 rpm for 5 min) and the superfluous medium was removed by pipetting. Consequently, 350 μL of ice-cold nuclei isolation buffer Otto I (0.1 M citric acid, 0.5% Tween 20; [39]) was added to the algal pellet, causing an osmotic rupture of cells and release of the sample nuclei. The resulting suspension was thoroughly shaken and kept on ice. Plants *Solanum pseudocapsicum* (2C = 2.59 pg [40]) or *Carex acutiformis* (2C = 0.82 pg [41]) were used as a (pseudo-)internal standard, depending on the sample DNA content. To release nuclei of the standard, ca. 20-mg piece of fresh leaf tissue was chopped with a razor blade in a plastic Petri dish with 250 μL of ice-cold Otto I buffer. Both suspensions (with algal and standard nuclei) were thoroughly mixed and filtered

through a 42 μm nylon mesh into a special 3.5-mL cuvette for direct use with the flow cytometer. Following a 20-min. incubation at room temperature, the sample was mixed with 1 mL of staining solution consisting of Otto II buffer (0.4 M $Na_2HPO_4{\cdot}12H_2O$; [39]), 50 μg · mL^{-1} PI, 50 μg · mL^{-1} RNase IIA and 2 μL · mL^{-1} β-mercaptoethanol. The stained sample was immediately analysed using a Partec CyFlow SL cytometer (Partec GmbH, Münster, Germany) equipped with a green solid-state laser (Cobolt Samba, 532 nm, 100 mW). In each sample, 5000 particles were measured and the resulting FCM histograms were analysed using FloMax ver. 2.4d (Partec). The first sample peak in the FCM histogram was identified as G_1 (vegetative cells) and a second peak with twice the relative fluorescence as G_2 (dividing cells). The absolute nuclear DNA content (C-value) was calculated as sample G_1 peak mean fluorescence / standard G_1 peak mean fluorescence × standard 2C DNA content (according to [42]). In case of low-quality measurements (i.e. G_1 sample peaks with coefficient of variation (CV) >5%), both sample preparation and analysis were repeated. To minimize the effect of random instrumental shift, each strain was analysed at least three times on separate days and the estimates averaged. Each time the three independent DNA content estimates differed by >3%, the most outlying measurement was discarded and a new measurement was carried out; however, the DNA content results of six strains (968, S20.45, S71.B4, V29, X40, K8) were averaged after five consecutive analyses with DNA content differences >3% (not exceeding 6%). Nuclear DNA content is reported in absolute units per cell (pg of DNA and equivalent values in Gbp). Since the chrysophytes are presumed to be haploids [22,43], when the DNA content variation within a species corresponded to two-fold differences, we referred to the lowest value as haploid (1x) and to its multiples as diploid (2x) or tetraploid (4x), with full awareness that the base ploidy level still needs to be verified. Despite our previous considerable effort, karyotyping and chromosome counts of various chrysophyte strains were not successful. To additionally corroborate the observed intraspecific ploidy level variation, a simultaneous analysis of three *S. glabra* strains (G11, F45 and L13), each representing a different ploidy level, was performed. Since eight strains indicated ploidy level change during cultivation, if possible, their cultures were repeatedly re-analysed (up to 8-times) within three consecutive years. In one strain of *S. petersenii* (C87), it appeared that three G_1 sample peaks differing in their ploidy level were present. Consequently, individual cells of this strain were inoculated into new subcultures (C87–1 - C87–6) and later (repeatedly) analysed for their DNA content. To avoid misinterpretation of G_2 peaks for a G_1 peak during the ploidy assignment, the presence of both G_1 and G_2 peaks was thoroughly checked and confirmed (usually clearly apparent in the relative fluorescence vs. side scatter plot) in all the analyses performed.

2.4. GC content estimation

To reveal a potential association between the nuclear DNA amount and a genome-wide proportion of GC bases, we analysed the genomic GC content of nine strains belonging to four *Synura* species (*S. americana*, *S. glabra*, *S. macropora*, *S. sphagnicola*). The strains were analysed using FCM with the AT-selective dye DAPI (4′,6-diamidino-2-phenylindole) and the results were directly compared with the PI FCM outputs for particular strains. We employed the same sample preparation as for PI FCM, except that the staining solution consisted of 1 mL of Otto II buffer, 4 μg · mL^{-1} DAPI and 2 μL · mL^{-1} β-mercaptoethanol. The stained samples were immediately analysed using a Partec PA II flow cytometer (Partec GmbH, Münster, Germany) equipped with a 488-nm UV LED as a source of excitation light. In each sample, 5000 particles were measured and the resulting FCM histograms were analysed using FloMax. Computation of the GC base content was done according to [44] via a publicly available Excel spreadsheet (http://sci.muni.cz/botany/systemgr/download/Festuca/ATGCFlow.xls). Each strain was analysed at least three times on separate days and the final estimate was averaged from the individual measurements.

2.5. Cell size measurements

In our search for phenotypic differences between intraspecific strains with different DNA contents we chose one trait – cell size. This trait is particularly important for unicellular organisms and a tight relationship between the cell size and nuclear DNA content has already been demonstrated (i.e. the nucleotypic effect [45]). We selected 12 strains belonging to five *Synura* species (*S. americana*, *S. glabra*, *S. macropora*, *S. petersenii,* and *S. sphagnicola*) exhibiting intraspecific ploidy level variation. Before the analyses, 50 μL of each strain at the exponential phase of growth was inoculated into 4 mL of fresh medium and cultivated for 2 weeks. After this period, microphotographs of individual cells were taken using a Leica DM2500 LED optical microscope with 40× magnification. The cell size was later estimated for each strain using ImageJ ver. 1.45 s [46] as object area on the microphotograph. The final estimates were based on a median value of 30 cells measured per each strain.

3. Results

3.1. Nuclear DNA content variation in chrysophytes

Altogether, this study was performed on 68 strains representing three chrysophyte genera, *Synura*, *Chrysosphaerella*, and *Ochromonas* (Supplementary data Table S1), where we previously detected intraspecific DNA content variation. Using the nuclear ITS rDNA (nu ITS rDNA) molecular barcode, we identified nine species of *Synura*: *S. americana, S. glabra*, *S. heteropora, S. hibernica*, *S. lanceolata*, *S. macropora*, *S. petersenii*, *S. soroconopea*, and *S. sphagnicola* (Fig. 1). Although the nu ITS rDNA showed minor sequence variations in several strains, this variability was not associated with DNA content differences. We successfully estimated absolute nuclear DNA contents for all strains. Intraspecific DNA content variation largely corresponded to the presence of different ploidy levels and was detected either in among-strain comparisons (Table 1; Fig. 2; for more detailed data, see Supplementary Table S2) or during repeated measurements on a single strain in cultivation (Figs. 3; 4; for more detailed data, see Supplementary Table S3). In each species, the lowest nuclear DNA content detected (for simplicity arbitrarily assigned as haploid; 1x) also had a corresponding diploid value (2x) with twice the DNA amount. A minor DNA content variation among strains in some species of *Synura* (Table 1, Fig. 1) did not compromise the overall pattern. The only exception was *S. macropora*, where higher DNA contents ranging 3.46–3.70 pg were not multiples of the lowest value (1.48 pg).

Ploidy level transitions during long-term cultivation were captured in seven strains of six chrysophyte taxa (*Chrysosphaerella* sp., *Ochromonas tuberculata*, *S. glabra*, *S. hibernica*, *S. lanceolata*, and *S. soroconopea*; Fig. 3 and 4; Supplementary Table S3). These transitions occurred in both directions, from haploid to diploid level and vice versa; multiple transitions suggesting alternation of the two ploidies over the course of time were observed in some strains. Moreover, the presence of three different ploidy levels (1x, 2x, 4x) was detected in two species (*S. glabra*, *S. heteropora*; Fig. 2; Table 1). Interestingly, different ploidy cytotypes in these two species even coexisted at one locality, within the same algal bloom. An initial indication of the presence of a tetraploid cytotype also in *S. petersenii* strain C87 was not corroborated and most likely represented G_2 of the diploid cells. When we re-inoculated individual cells from the original colonies and established six subcultures, they became fixed for either haploid or diploid ploidy level. Interestingly, repeated analyses of *S. petersenii* strain C87 subcultures did not show any further ploidy transitions over the period of two years.

3.2. Genomic GC content

The genomic GC content ranged from 38.0 to 47.7% among nine strains representing different ploidy levels of four *Synura* species

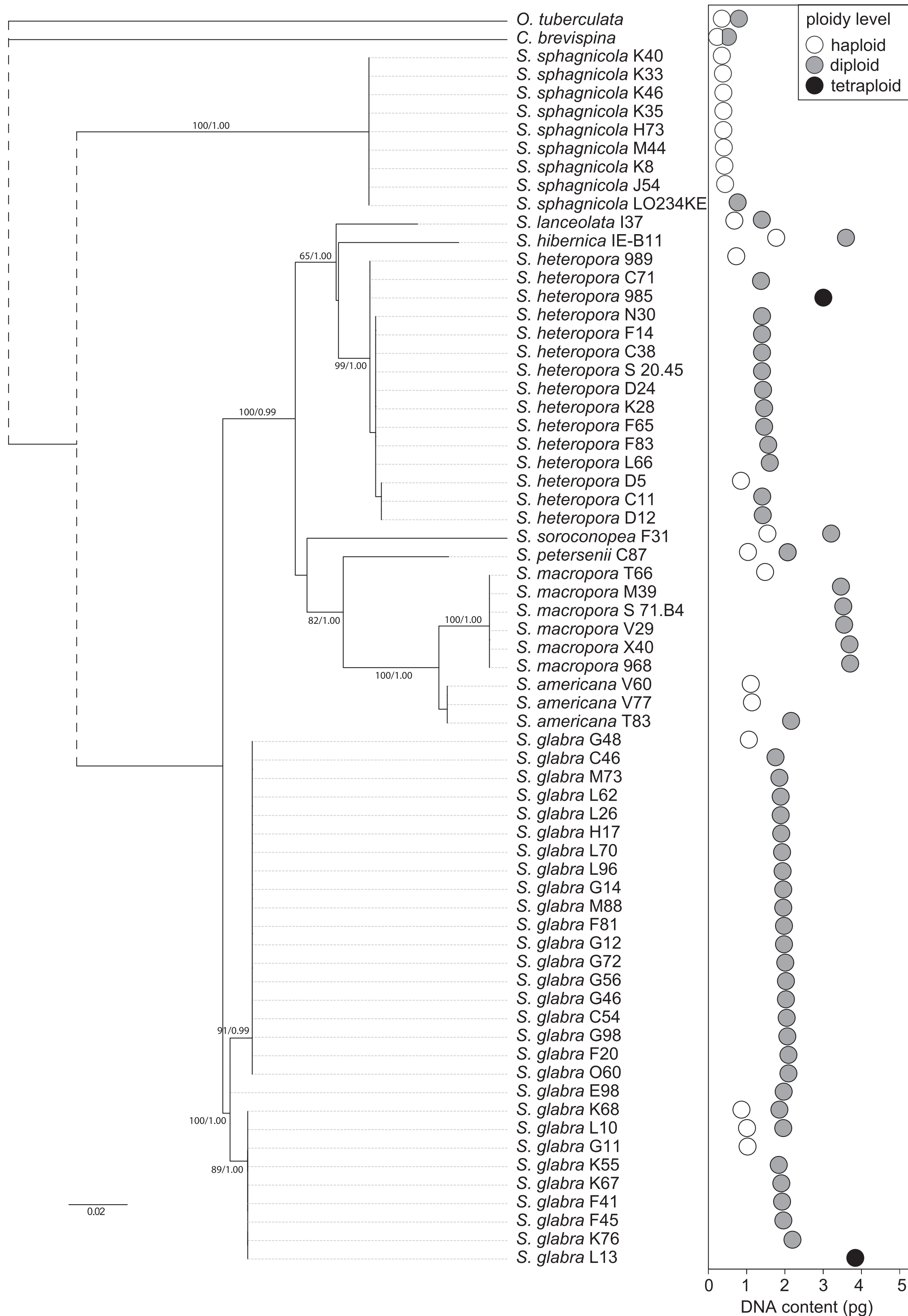

Fig. 1. Phylogenetic relationships and DNA contents of investigated chrysophyte strains. Maximum likelihood (ML) phylogeny of nine *Synura* species is based on ITS rDNA sequences. Values at the nodes indicate statistical support estimated by – ML bootstrap (left) and MrBayes posterior node probability (right). Only statistical supports higher than 60/0.95 are shown. Scale bar – estimated number of substitutions per site. The phylogenetic relationships of the species *Ochromonas tuberculata* and *Chrysosphaerella brevispina* are illustrated by dashed line following phylogenetic analysis in Kristiansen and Škaloud [17]. The strains possess various DNA contents from 0.23 to 3.83 pg and haploid (white), diploid (grey) or tetraploid (black) ploidy level.

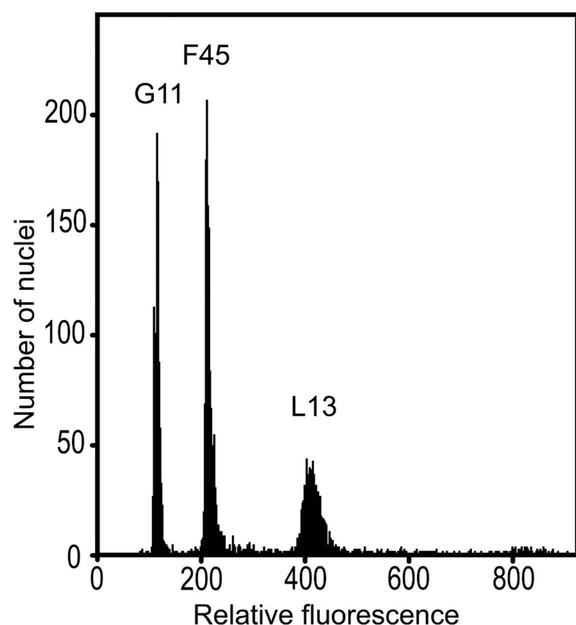

Fig. 2. A simultaneous flow cytometric analysis of three strains of *Synura glabra* that differ in their ploidy level (G11 - haploid, F45 - diploid, and L13 - tetraploid). Nuclei were stained with propidium iodide.

(*S. americana*, *S. glabra*, *S. macropora, and S. sphagnicola*). In general, the GC content was highly similar for intraspecific ploidy cytotypes, despite the multiple-fold difference in their DNA content (Table 2). Specifically, the GC content values of strains ranged from 39.6 to 40.8% in *S. americana*, from 38.0 to 38.6% in *S. glabra*, and from 47.3 to 47.7% in *S. sphagnicola*. A marked exception were *S. macropora* strains exhibiting increased GC content variation 38.3–42.2%.

3.3. Phenotypic consequences of ploidy level variation

Ploidy level increase (i.e., doubling of nuclear DNA content) was associated with greater cell size in the investigated chrysophyte taxa (Table 2). In intraspecific comparisons, shifts from haploid to diploid ploidy level resulted on average in the cell size increase by 34% (range 13–55%). This trend was observed both among different strains of the same species and among haploid and diploid subcultures of the same strain (in the case of *S. petersenii* strain C87). Interestingly, except for the change in cell size, the overall phenotypes of different ploidy levels appeared to be the same (see Figs. 5 and 6).

4. Discussion

4.1. Intraspecific ploidy level variation in chrysophytes

With the exception of a single species (*S. macropora*, see below), DNA content variation within and among conspecific strains was not random but corresponded to different ploidy levels. Two to three ploidy levels were detected within the particular chrysophyte species investigated, here for simplicity referred to as haploid (1x), diploid (2x) and tetraploid (4x).

Aside from the (almost) exactly two-fold differences in nuclear DNA contents, the intraspecific ploidy level variation was also supported by the highly similar genomic base composition (GC content), a trait that otherwise spanned rather broadly among the species (38.0–47.7%). Moreover, we are convinced that our records of the higher ploidy levels (2x, 4x) are not simply artefacts caused by the occurrence of dividing cells in G_2 phase of the life cycle (i.e., after duplication of genomic DNA but not yet entering mitosis). Before assigning a ploidy, we always checked for the presence of at least a small population of nuclei with twice the fluorescence intensity (~ DNA content) than our peaks of interest in flow cytometric histograms, that correspond to G_2-phase cells [47]. Key insights into the evolutionary processes responsible for the observed DNA content variation were provided by long-term cultivation of strains, when we detected alternation between the lower and higher ploidy states over time (Fig. 4). This suggests that ploidy level shifts are an inherent part of the chrysophytes' life cycles. Additionally, our records of three different ploidy levels in two species of *Synura* (*S. glabra*, *S. heteropora*) suggest that part of the DNA content diversity is also contributed by polyploidization.

While most (minor) deviations from the two-fold differences in nuclear DNA contents can be attributed to the error of measurement or to the higher content of secondary metabolites interfering with DNA staining, some might have arisen from genetic differentiation of strains during evolution. A prominent case of the latter are the strains of *S. macropora* with the DNA contents of either 1.5 pg or 3.6 pg, which also

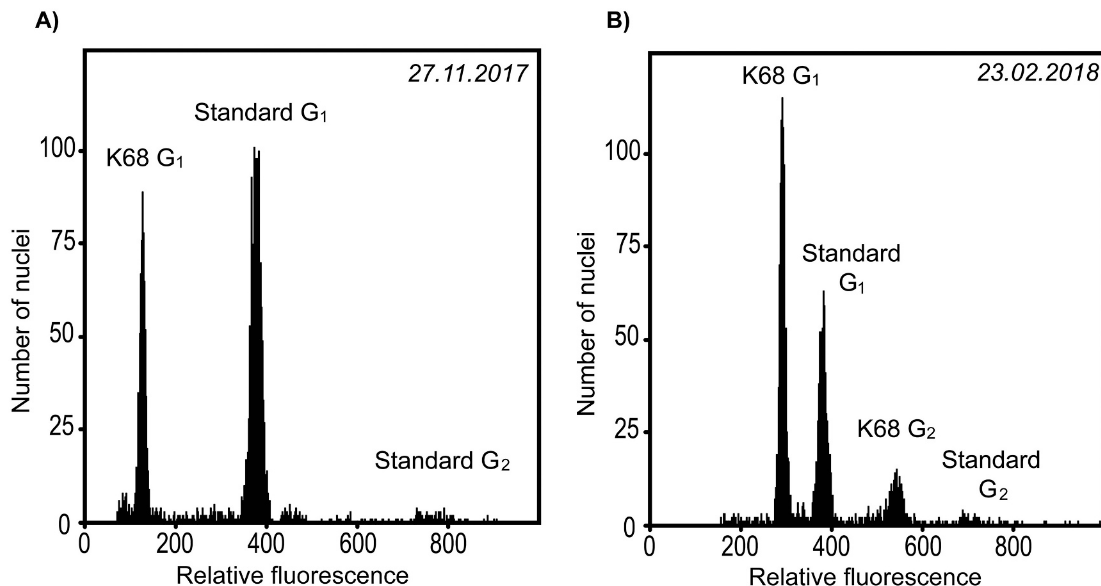

Fig. 3. A life cycle stage transition and the inherent ploidy shift from haploid (A) to diploid (B) level documented in *Synura glabra* strain K68 by two consecutive flow cytometric measurements. Plant *Solanum pseudocapsicum* was used as a reference standard.

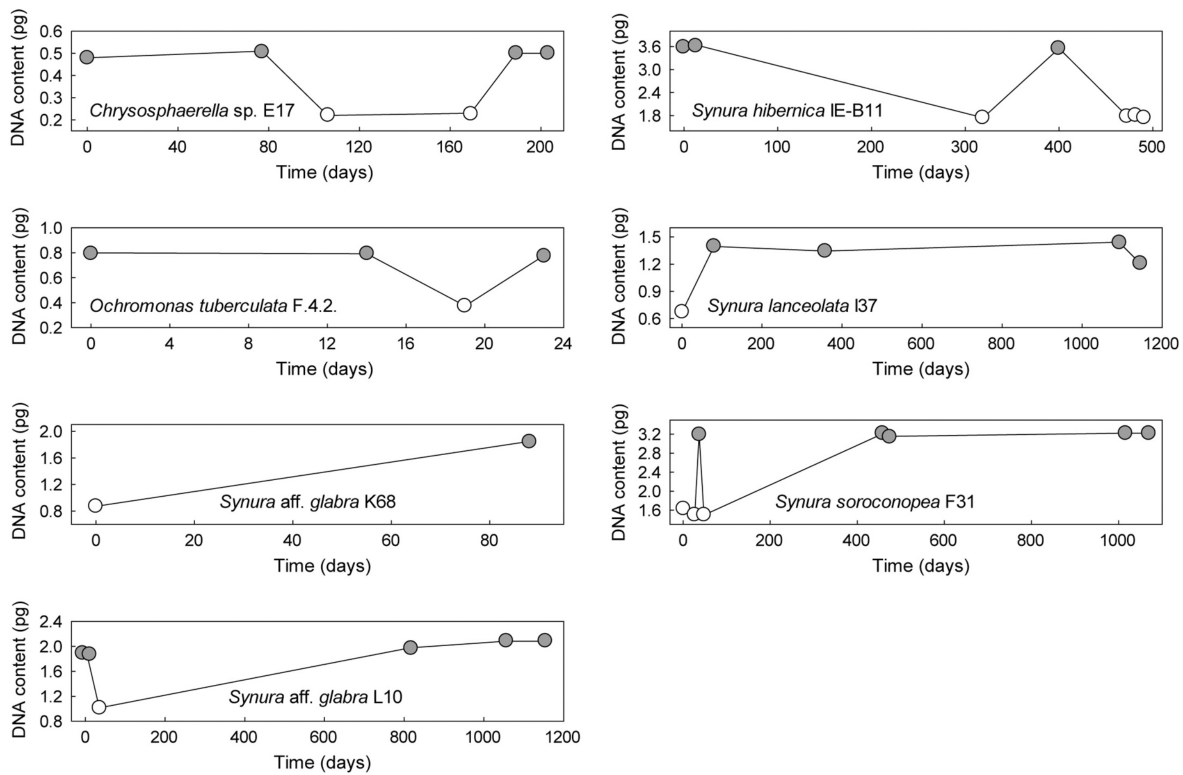

Fig. 4. Two-fold differences in nuclear DNA content captured by a time series of flow cytometric measurements on seven strains of chrysophyte taxa alternating between the haploid (white) and diploid (grey) life cycle stage.

Table 1
Nuclear DNA content and ploidy level variation among chrysophyte species. Ploidy level assignment is arbitrary, assuming the lowest DNA content category in each species corresponds to a haploid. The predominant ploidy level in each species is marked with an asterisk.

Species	No. of strains	Nuclear DNA content [pg; median ± SD]		
		1x ploidy level	2x ploidy level	4x ploidy level
Chrysosphaerella brevispina	1	0.2	0.5*	
Ochromonas tuberculata	1	0.4	0.8*	
Synura americana	3	1.1 ± 0.02*	2.2	
glabra	27	1.0 ± 0.02	2.0 ± 0.09*	3.8
heteropora	15	0.8 ± 0.06	1.4 ± 0.07*	3.0
hibernica	1	1.8*	3.6	
lanceolata	1	0.7	1.4*	
macropora	6	1.5	3.6 ± 0.09*	
petersenii	1	1.0	2.1*	
soroconopea	1	1.5	3.2*	
sphagnicola	9	0.4 ± 0.03*	0.8	

have distinct genomic GC contents (Table 2).

These strains could represent different ploidy levels of distinct lineages (cryptic taxa) within *S. macropora*, though their evolutionary differentiation must have occurred relatively recently given that the nu ITS rDNA region has not yet diversified (see Fig. 1). Similarly, pronounced shifts in nuclear DNA content not followed by the nu ITS rDNA diversification were already described among *S. petersenii* strains [23].

4.2. Life cycle of chrysophytes

Even though the chrysophytes with over 1100 described species [48] constitute a relatively large group of microscopic algae, the knowledge of their life cycles is very fragmentary. There are few instances in the older literature describing the formation of gametes and their fusion [18,20,21], but neither the sexual reproduction in chrysophytes, nor other key aspects of their life cycle have been since then a subject of targeted study. Our flow cytometric data has clearly shown that the life cycle of chrysophytes involves alternation between a lower and a higher ploidy state. As a consequence, ploidy level shifts may occur within a

Table 2
Genomic GC content and cell size estimates for selected strains of *Synura* exhibiting intraspecific ploidy level variation.

Species	Strain	Ploidy level	DNA content	GC content [mean ± SD]	Cell size [mean ± SD]
S. americana	V77	1x	1.13 pg	39.6 ± 0.1%	101 ± 23 μm²
	T83	2x	2.16 pg	40.8 ± 0.2%	114 ± 10 μm²
S. glabra	G11	1x	1.02 pg	38.6 ± 0.3%	80 ± 12 μm²
	K67	2x	1.91 pg	38.4 ± 0.2%	124 ± 21 μm²
	L13	4x	3.83 pg	38.0 ± 0.2%	137 ± 23 μm²
S. macropora	T66	1x	1.48 pg	38.3 ± 0.2%	139 ± 25 μm²
	S71.B4	2x	3.53 pg	42.2 ± 0.2%	99 ± 13 μm²
S. petersenii	C87–6	1x	1.08 pg	–	104 ± 11 μm²
	C87–2	2x	2.07 pg	–	139 ± 11 μm²
	C87–5	2x	2.12 pg	–	157 ± 17 μm²
S. sphagnicola	K35	1x	0.39 pg	47.3 ± 0.0%	112 ± 21 μm²
	LO234KE	2x	0.76 pg	47.7 ± 0.1%	132 ± 24 μm²

Fig. 5. Phenotypes of haploid, diploid and tetraploid strains of *Synura glabra*, mainly differing by the size of cells in their colonies. Scales = 10 μm.

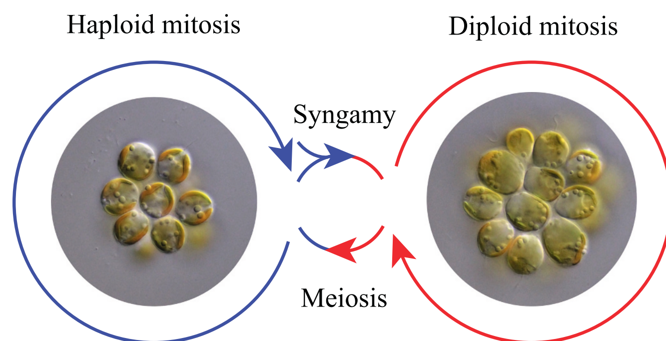

Fig. 6. An illustrative scheme of the isomorphic haploid-diploid life cycle of chrysophytes. Both haploid and diploid stages are capable of vegetative reproduction and have a more-or-less uniform phenotype (shown here on example of *S. petersenii* strains C87–6 and C87–2). Syngamy refers to the fusion of haploid gametes.

single strain maintained in cultivation, and independently collected strains of a single species may exhibit ploidy level variation. Moreover, based on repeated DNA content measurements conducted on strains under long-term cultivation, we are convinced that the lower ploidy state is not restricted to short-lived gametes and the higher ploidy state is not equivalent to a zygote. The chrysophyte cells at either ploidy state are able of mitotic propagation which allows them longer-term persistence in cultivation. Our findings are thus consistent with chrysophytes having a haploid-diploid life cycle. This life cycle was already proposed for several groups of unicellular algae, namely for haptophytes, foraminifera, some dinoflagellates and some cryptophytes [4,8,9].

As a general rule, different life cycle stages in these groups are morphologically well distinguishable. For example, in the haptophyte species *Emiliania huxleyi*, diploids are covered by prominent calcified scales and lack flagella, while flagellated haploids are covered by inconspicuous organic scales [49]. In another haptophyte species, *Phaeocystis globosa*, haploids and diploids differ by the presence of organic scales [8]. Different life cycle stages of the cryptophyte *Cryptomonas* were even previously described as two separate genera, *Cryptomonas* and *Campylomonas* [50]. In chrysophytes, this does not seem to be the case and different life cycle stages are morphologically indistinguishable. Apart from a small increase in cell size with the higher ploidy level, we have not observed any apparent phenotypic differences between the two stages. Hence, the chrysophytes have an isomorphic haploid-diploid life cycle (see Fig. 6 for a scheme). Interestingly, it is the first record of this life strategy among unicellular algae. There are two consequences that can be derived from the life cycle of chrysophytes. First, even though capable of long-term persistence via mitotic propagation, the haploids must also play the role of gametes. Second, the observed ploidy level shifts indicate that diploid strains regularly undergo meiosis in cultivation. In spite of the latter, we never directly observed the sexual reproduction nor have identified preferred conditions under which it occurs.

Questions arise as to how often life stage transitions occur, what triggers them, and whether chrysophyte species spend more time as haploids or diploids. Our results suggest that, at least ex situ, under cultivation, life cycle transitions may occur very rapidly. For example, in *Ochromonas tuberculata* and *Synura soroconopea*, two ploidy level shifts were observed within two weeks. On the other hand, most other species seemed to alternate in ploidy considerably more slowly (Fig. 4). It should be noted though, that intervals in our time series of flow cytometric measurements were highly irregular as ploidy level variation within strains was usually first detected only by coincidence. Consequently, it is very likely that some life stage transitions have remained undetected in our long-term cultivation experiment. Also, the strains and taxa used in this study were specifically selected from a much broader collection based on the intraspecific DNA content variation that was detected during our ongoing research. It seems the probability of detecting the life stage transitions is rather low, though it does not necessarily mean such transitions are rare in natural populations of chrysophytes.

Interestingly, since each flow cytometric measurement was conducted on thousands of cells, our results also prove that all cells in each culture had synchronous life cycles. The only exception was an initial coexistence of haploids and diploids in *S. petersenii* strain C87, but soon after their re-inoculation, the life cycles synchronized and six resulting subcultures became fixed for either the haploid or the diploid stage. Possibly, the first measurement might have caught the cells in the middle of a life stage transition. It can be expected that such synchronous life stage transitions are also applicable to individuals within natural populations. In general, chemical signalization among cells could be associated with the life stage transitions in chrysophytes, ensuring they enter particular life cycle stages or produce their gametes synchronously. Production of pheromones, allowing synchronization of individuals prior to the mating process was already described in green algae (e.g. *Volvox carteri* [51]). Similarly, chemical signalling is involved in sexual induction of some diatoms [52]. When we summarize our DNA content measurements across the studied chrysophyte species (Table 1, Fig. 4), it seems that the diploid life cycle stage predominates in most, though there are also exceptions where the haploids are more common (i.e., *Synura americana*, *S. hibernica* and *S. sphagnicola*). This suggests that the relative duration of these two stages in the life cycles of chrysophytes could differ from taxa to taxa, possibly reflecting their environmental preferences and/or population genetic processes.

4.3. Evolutionary benefits of a haploid-diploid life cycle

It is widely believed that the differentiation of life cycle on two alternating ploidy phases comes with many evolutionary advantages. From a genetic point of view, the haploid stage allows more efficient selection of beneficial alleles and immediate elimination of deleterious

mutations, whereas the diploid stage has the ability to accumulate mutations at a higher rate and mask deleterious mutations, resulting in increased genetic diversity [1,53]. From an ecological perspective, the existence of two or more life cycle stages may provide for more efficient specialization, better exploitation of the environment, and thus increasing the evolutionary success of species [54]. The evolution and maintenance of haploid-diploid life cycles was nicely demonstrated by Hughes and Otto [55]. Using a genetic model parametrized with demographic data of red algae *Gracilaria gracilis*, the authors conclude that haploid-diploid life cycles may be evolutionarily stable as long as resource competition between haploids and diploids is sufficiently weak. As an example from microscopic algae, the populations of haptophyte *Emiliania huxleyi* often suffer from infections by phycodnaviruses. However, the transition from heavily calcified susceptible diploids to resistant haploids lacking calcified covering serves as an escape mechanism [56]. Interestingly, even subtle phenotypic differences between the two stages in isomorphic species may have significant ecological consequences [55,57,58].

The only apparent difference we have observed between the phenotypes of conspecific haploids and diploids was a small increase in cell size. A positive association between the ploidy level and cell size was consistently observed in all chrysophyte species (see Table 2), in spite of a considerable variation in cell shapes due to the lack of a cell wall (Fig. 5). Increase in cell size is actually the most common phenotypic effect of higher ploidy and it is usually considered to be a direct consequence of the doubling of nuclear DNA content [45,55]. Particularly in unicellular organisms, the cell size is one of the most important traits because it fundamentally relates to metabolic rate, growth rate or generation time, but can also affect species temperature optima, dispersal abilities, or susceptibility to herbivores [59–61]. As a consequence of their geometry, larger cells have lower surface area to volume ratio than small ones of identical shape. Thus, smaller haploid cells with a relatively higher surface area should be more efficient in nutrient uptake and at the same time, they may require lower energetic costs to maintain their life functions and have faster cell division [55,62]. The "nutrient limitation hypothesis" by Lewis [62] then predicts that haploids will better exploit low-nutrient environments, and this should be especially applicable to unicellular planktonic autotrophs (e.g., chrysophytes). On the other hand, diploids might be preadapted to better tolerate toxic environments due to their relatively smaller surface area interacting with the external environment [1].

As was already mentioned above, even though chrysophytes can persist in either haploid or diploid stage in cultivation, the diploids seem to prevail among strains and taxa. We can only speculate that nutrient-rich environments, supplemented in our study with cultivation medium, may favour the diploid life cycle stage in line with Lewis's hypothesis [62]. Since both stages are part of a one life cycle and principally should alternate in populations, any putative associations between the occurrence of haploids / diploids and environmental conditions will likely reflect temporal (e.g. seasonal) changes in the suitability of particular habitats for chrysophyte taxa. From this perspective, it would be interesting to observe life stage transitions in chrysophyte cultures being induced by the input of a fresh medium (although our preliminary observations do not support it, data not shown). On the other hand, the cultures reaching a certain "population density" could also serve as a trigger, and a particularly important one in the terms of potentially forecasting a bloom formation.

We believe that due to their isomorphic haploid-diploid life cycle, unicellular chrysophytes may serve as a unique model to answer various questions related to the evolutionary importance of ploidy level variation among unicellular algae, and the potential benefits and costs of having different ploidy phases in a life cycle or maintaining diploid and polyploid cytotypes. These will be the subject of our further investigation.

4.4. DNA contents and polyploidy in chrysophytes

The available data shows that there is a considerable variation in nuclear DNA contents among chrysophytes, equalling to a 276-fold difference between *Segregatospumella dracosaxi* with the smallest reported DNA content and *Mallomonas caudata* with the largest DNA content (0.09 pg and 24.85 pg, respectively [22,23,63]). While the nuclear DNA content variation might have played an important role in the chrysophyte evolution, DNA content estimates are still available for only a small fraction of the overall species richness in Chrysophyceae (32 species ~3%). In addition to the insights into the chrysophytes' life cycles, this study provides a large number of DNA content estimates. Here, we provide the first DNA content estimates for the genus *Chrysosphaerella* and the species *Ochromonas tuberculata*, *Synura glabra*, *S. hibernica*, *S. lanceolata*, *S. macropora*, and *S. soroconopea*. Our estimates for the species *S. petersenii* (1.04 / 2.09 pg) fall well within the previously described wide range of intraspecific DNA contents [23]. The previously published estimates for two strains of *S. heteropora* (S 20.45, WA18K-A) are 1.51 pg and 1.55 pg, respectively [22], which is in agreement with our estimates for 12 strains (mean = 1.44 pg, range = 1.38–1.61 pg) and also confirms the overall prevalence of the diploid life cycle stage. Similarly, with the exception of a single strain (L0234KE), our DNA content estimates for eight *S. sphagnicola* strains corroborate the previously published data (0.40 pg; [22]). Note that Olefeld et al. [22] considered that chrysophytes are presumably haploid, but when providing a 1C value, the authors divided the estimated DNA contents by two as if they were diploids. On the other hand, the DNA content of *S. sphagnicola* strain L0234KE, introduced as a flow cytometric (FCM) standard in Olefeld et al. (2018), was referred to as 0.40 pg, while our estimate for the same strain is 0.76 pg. This inconsistency most likely results from a life stage transition of the strain between the two measurements. Even though the best practice is to apply closely related species as FCM standards [64], our findings show that the use of chrysophyte FCM standards should be highly discouraged due to their isomorphic haploid-diploid life cycle and the resulting DNA content instability.

In addition to the haploid – diploid life stage transitions, the presence of a third, higher ploidy detected in two chrysophyte species (*S. glabra* and *S. heteropora*) suggests the involvement of polyploidy. The tetraploid cytotype was quite rare, comprising just 3% of the investigated strains. Both the precisely two-fold DNA content difference from respective diploids and ITS rDNA homogeneity favour their autopolyploid origin (i.e., intraspecific polyploidy). At one locality of *S. heteropora*, the tetraploids even coexisted with haploids in a mixed-ploidy population (strains no. 985 and 989; Vltava river, Prague, Czech Republic). A similar coexistence of *S. glabra* diploids and tetraploids in Inari lake (strains K76 and L13–2; Inari, Finland) could either represent the lower- and higher-ploidy life stages of a single clone, or belong to two different clones, both at the higher-ploidy stage. We can hypothesize that such coexistence of different ploidy cytotypes in a population can be maintained by their prevalent asexual reproduction via mitotic division. The polyploidy was recently observed in another chrysophyte, *Poteriospumella lacustris* [65], and is likely a recurrent phenomenon in chrysophytes.

5. Conclusions

In this study, we revealed that chrysophytes have a haploid-diploid life cycle. Chrysophyte taxa alternate between two ploidy states, both of which are capable of mitotic propagation and long-term survival in cultivation. The two life cycle stages are morphologically undistinguishable, apart from a small increase in cell size with the higher ploidy level. This is the first report of an isomorphic haploid-diploid life cycle among unicellular algae. Interestingly, our flow cytometric measurements also revealed that life cycles are well synchronized among cells coexisting within a culture and the same can be expected for

natural populations of chrysophytes. The relative duration of the life cycle stages may differ from taxa to taxa. While the diploid stage prevailed in most of our studied chrysophyte representatives, in others the haploid stage seemed to dominate. Collectively, our results provide new unique insights into the chrysophyte life cycle. The chrysophytes may serve as a suitable model for studying the benefits and costs of having different ploidy stages in a life cycle.

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CRediT authorship contribution statement

Dora Čertnerová: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Writing – original draft. **Martin Čertner:** Funding acquisition, Writing – review & editing. **Pavel Škaloud:** Resources, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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