University of South Bohemia

Faculty of Science

Study on the chromosome number in the alveolate alga *Chromera velia* by TSA-FISH

Master thesis

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Annotation:
This thesis is focused on the chromosome-number studies in alveolate alga *C. velia*. This Apicomplexa-related photosynthetic organism proved to be a unique instrument for the study of Apicomplexa and their unique organelle apicoplast. To better understand the similarities and differences within these two phyla, a further chromosome analysis was needed. The first part of this thesis sums up the present knowledge about ploidy, life cycle and genome organization within *C. velia* and the close-relative phyla of Apicomplexa and Dinoflagellata. The second part describes our attempt to examine the ploidy and total number of chromosomes in *C. velia* using fluorescence *in situ* hybridization. The ploidy of *C. velia* was successfully determined and we also made a significant progress in the determination of the total chromosome number.

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Contents

List of abrreviations

b, kb, Mb, bp: base, kilobase, megabase, base pair

cox: Cyclooxygenase or prostaglandin-endoperoxide synthase

CP: *Cydia pomonella*

CV: *Chromera velia*

cytb: Cytochrome b

DAPI: 4',6-diamidino-2-phenylindole

dATP, dUTP: 2'-deoxyadenosine 5'-triphosphate, 2´-Deoxyuridine, 5´-Triphosphate

DIG: Digoxigenin

DNP: Dinitrophenol

FISH: fluorescence *in situ* hybridization

gDNA: genomic DNA

HRP: Horseradish peroxidase

LLYC: lysozyme, lysing enzymes, celullysine

PCR: polymerase chain reaction

PS: *Pisum sativum*

psbA: photosystem II protein D1

RT: room temperature

SC DNA: SC-pGEM plasmid DNA

SC: single copy (gene)

SSU rRNA: small-subunit ribosomal RNA

T DNA: telomere DNA

TELO: telomere

T_m: melting temperature

TSA-FISH: tyramide signal amplification – fluorescence *in situ* hybridization

1. Introduction

1.1 *Chromera velia* – discovery and phylogenetic classification

Chromera velia is a unicellular autotrophic green-brown alga that was accidentally discovered in 2001 during the attempts to isolate *Symbiodinium* – the most significant symbiont of stony corals *Plesiastrea versipora* – in Sydney Harbor, New South Wales, Australia (Moore et al., 2008; Oborník et al., 2012). Within its life cycle, *C. velia* occurs in the five distinctive forms – a vegetative coccoid cell, an autosporangium, a cyst, a zoosporangium and a zoospore/flagellate (three of them see in Fig.1). The dominantly occurring form of *C. velia* is the immobile vegetative coccoid cell which is brownish, spherical and app. 5 to 7 μm in diameter large. The second most frequent is up to 9.5 μm sized autosporangium which is consisted of two, three or four vegetative coccoid cells covered with additional thin external membrane. The third principal stage is the zoospore formed inside the zoosporangium (Oborník et al., 2015). In this stage, the cells are egg-shaped, 5 × 3 μm in size, and equipped with two heterodynamic flagella enabling them to move. The cysts occur predominantly in adverse conditions (Moore et al., 2008; Oborník et al., 2011; Weatherby et al., 2011).

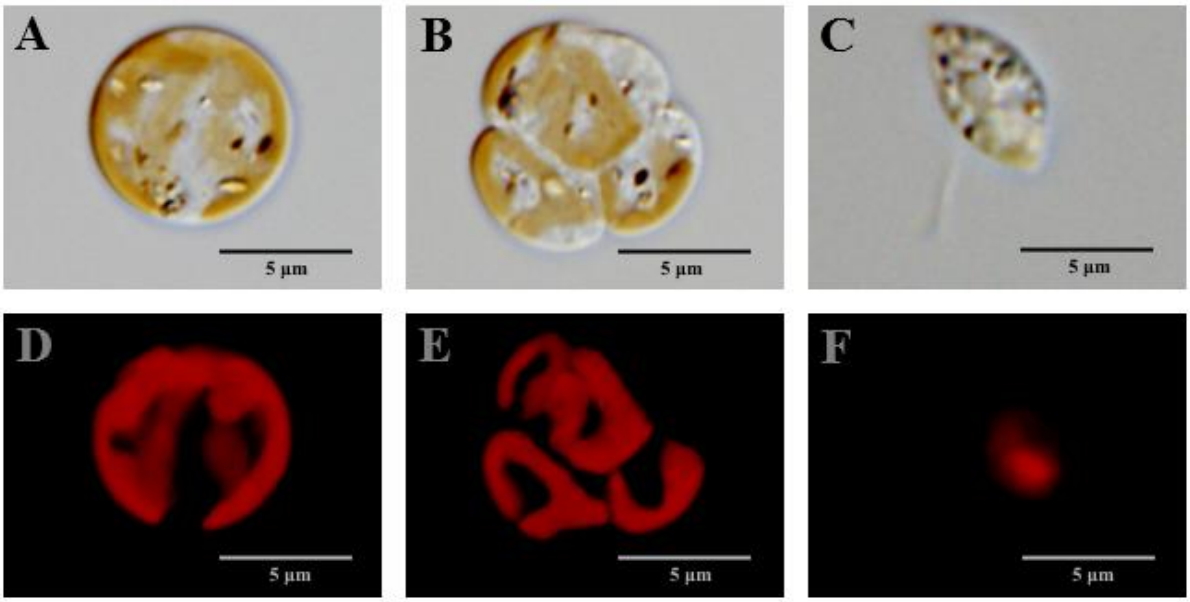

Fig.1: The three common forms of *C. velia* – a coccoid cell (A, D), an autosporangium (B, E), and a zoospore/flagellate (C, F). Upper series – light microscopy, bottom series – fluorescence microscopy (autofluorescence of the plastid). (Oborník et al., 2015).

At the time of the discovery of *C. velia*, another organism was described to associate with *Leptastrea purpurea* from One Tree Island, Great Barrier Reef, Queensland, Australia – an alga subsequently named *Vitrella brassicaformis* (Moore, 2006; Moore et al., 2008; Oborník et al., 2012). On the basis of molecular phylogeny, similar metabolic features, and photosynthetic ability, both *C. velia* and *V. brassicaformis* were unified into a novel phylum *Chromerida*. However, there is a substantial variation in morphology and molecular characters placing them into separate families – *Chromeraceae* for *C. velia* and *Vitrellaceae* for *V. brassicaformis.* Further phylogenetic analyses of the plastid SSU rRNA genes, as well as the entire plastid genome, affirmed inclusion of chromerids into the species-diverse group Alveolata and demonstrated their unexpected affiliation to colpodellids – poorly studied free-living heterotrophic predators of algae – and relation to dinoflagellates and, surprisingly, to parasitic Apicomplexa (Janouškovec, et al., 2015).

Colpodellids (including *Chromera* and *Vitrella*), dinoflagellates and apicomplexans together form the subgroup Myzozoa (see Fig.2) (Ruggiero et al., 2015). Dinoflagellates are of high ecological importance algae living in sea, brackish, and even fresh water environment, where approximately a half of them are photosynthetic (Adl et al., 2012). The typical representative of this phylum is aforementioned *Symbiodinium*, well known as important symbiont of corals (Freudental, 1962; Trench, 1993). The phylum Apicomplexa includes protists parasitic on various representatives of Metazoa ranging from polychaetes (Rueckert et al., 2009) to humans (Smith, 1996; Duszynski et al., 1999). For instance, *Plasmodium falciparum* is well known to cause malaria – the most devastating parasitic disease of humans, while *Toxoplasma gondii* is probably the most widespread human parasite with presumed behavioral influence on the host (Flegr, 2007).

Despite obvious differences in appearance and lifestyle, Myzozoa do share morphological traits as a certain form of an apical complex. Most importantly, Apicomplexa contain a distinctive organelle referred to as the apicoplast. The apicoplast evolved from rhodophyte-derived secondary plastid after losing its ability of photosynthesis, but it still retains essential biochemical functions. Based on genome sequence, the apicoplast is closely related to the photosynthetic plastid of *C. velia* and the peridinin plastids of dinoflagellates (Moore et al., 2008; Oborník et al., 2009; Janouškovec et al., 2010; Kořený et al., 2011). Also other phylogenetic analyses placed chromerids as the closest phototrophic relatives to Apicomplexa, capturing the blank space between these obligatory parasites and the free-living dinoflagellates (Moore et al., 2008; Oborník et al., 2009; Janouškovec et al., 2010;

Burki et al., 2012; Janouškovec et al., 2015; Woo et al., 2015). Therefore, *C. velia* represents a unique opportunity to study an apicoplast-related functional chloroplast and reconstruct its transition into a non-photosynthetic organelle. Furthermore, studying a free-living relative allows reconstruction of the ancestral cellular features the apicomplexans had before switching to parasitism.

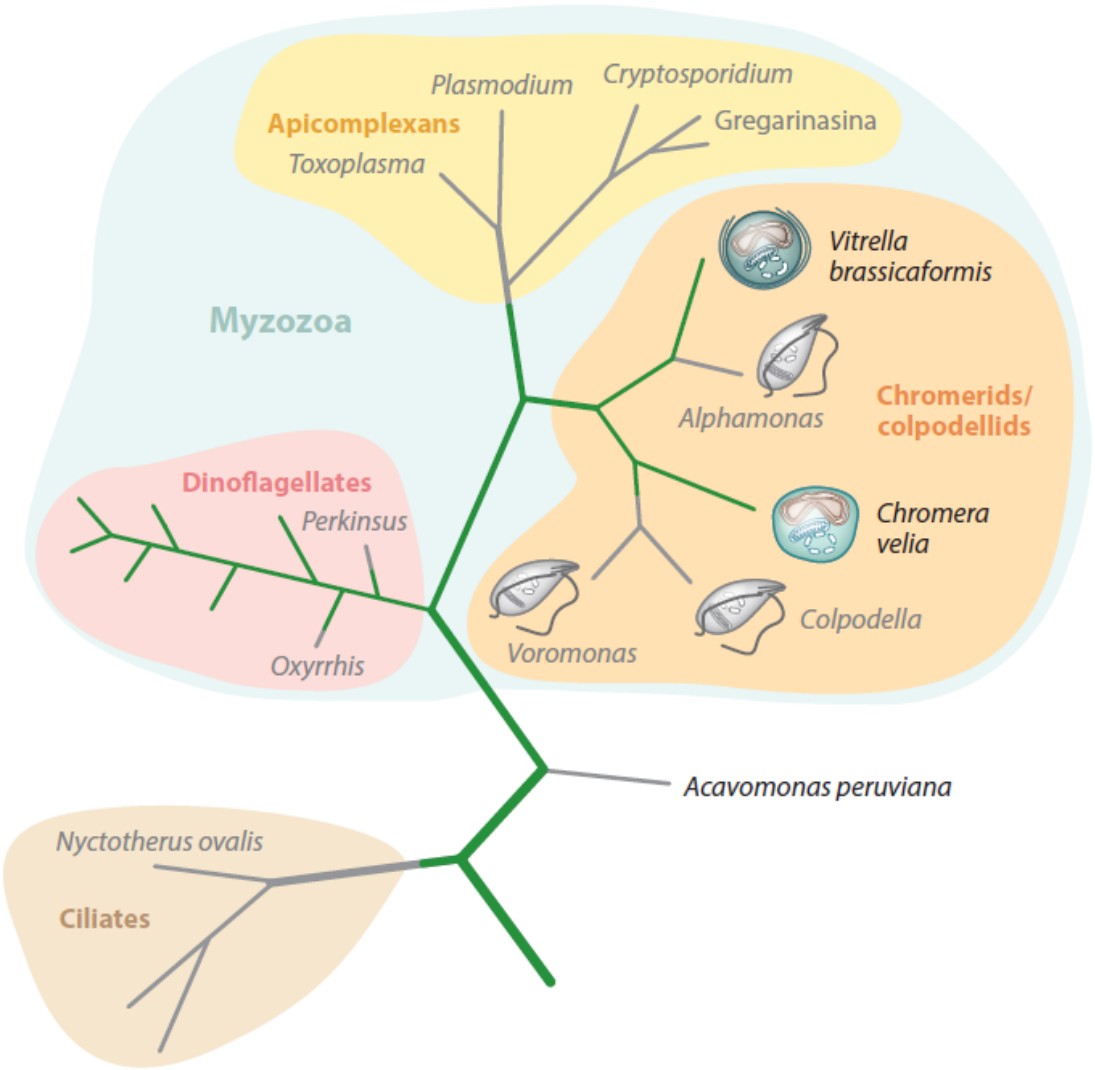

Fig.2: The phylogenetic tree representing the position of *Chromera velia* within the subgroup of Myzozoa and the group of Alveolata (Oborník and Lukeš, 2015).

1.2. Ploidy and evolution (role in selection)

Ploidy, i.e. the number of sets of chromosomes in a cell, can momentously affect genome evolution. One would assume that diploids, containing twice as much DNA as haploids, may accumulate new beneficial mutations at a higher rate (Paquin and Adams, 1983). An additional copy of an allele in diploids could present a noticeable benefit, because while the

original function is maintained by one allele, the second allele would be open to gain a novel function (Lewis and Wolpert, 1979). In contrast, manifestation of a new mutation may be reduced in diploids because of partial masking. In other words, allele fixation or disposal is more efficient and rapid in haploids, because all mutations are instantly exposed to selection, while detrimental phenotypic effects can be masked by the dominant allele in heterozygote diploids (Orr and Otto, 1994; Hughes and Otto, 1999; Otto and Gerstein, 2008). On top of that, adaptation is faster in haploids and its rate is limited by selection (Zeyl et al., 2003; Gerstein et al., 2010). The predictions of the masking theory were confirmed in experiment where unicellular diploids showed lower sensitivity to mutagen and slightly slower fitness recovery after the mutagen treatment, as compared to haploids (Mable and Otto, 2001). Similarly, in organisms with haploid-diploid alternation within their life cycle, beneficial mutations are fixed and deleterious mutations are disposed of more effectively in genes with expression restricted to the haploid phase (Bell, 2008). In summary, haploid cells seem more suited for adaptation in changing environments.

1.3. Ploidy and life cycle in Myzozoa

Although there is a relatively considerable variation in the life cycle within Myzozoa, as for ploidy, Apicomplexa and dinoflagellates are primarily haploidic with interim diploidic stadium (Taylor, 1987; Margulis, 1990). The ploidy of colpodellids, the closest relatives of *C. velia*, as well as the ploidy of *C. velia* itself, has not yet been described.

1.3.1. Ploidy and life cycle in Apicomplexa

Complex life cycles with diverse cellular forms in different stages are characteristic for the strictly parasitic species of phylum Apicomplexa (Margulis, 1990; Nuismer and Otto, 2004). In brief, four cell stages are usually needed to complete the cycle – a zygote, a sporozoite, a merozoite and a gametic stage. First, a diploid zygote is formed inside a cyst after the fusion of male and female gametes. The encysted zygote enters a new host and meiotically divides, forming haploid sporozoites. Inside the host body, sporozoites infect and enter specific cells using binding antigens and surface proteins for their recognition and penetration (Baum et al., 2008). Sporozoites then transform to a multinucleate body (the meront) to asexually produce haploid merozoites. The haploid sexual gametes are then formed by differentiation, and fertilization occurs to yield a zygote, completing the cycle. (Levine, 1973; Lee, 2000). The typical series of sexual and asexual reproduction takes place either entirely in one

animal host, or alternating between two hosts is necessary and different stages are specific for each host.

1.3.2. Ploidy and life cycle in *Chromera velia*

Based on the latest data, *C. velia* is considered a facultative symbiont of corals. Presence of *C. velia* cells was shown on the coral surface, suggesting epiphytic growth, (Janouškovec et al., 2012a,b) but surprisingly also inside the tissue of *Acropora digifera* and *A. tenuis* larvae, indicating a symbiotic relationship (Cumbo et al., 2013). Sexual reproduction as well as the ploidy of *C. velia* have not yet been described. Only asexual reproduction has been observed in *C. velia* suggesting a vegetative life cycle (Fig.3, Oborník et al., 2011; Oborník et al., 2012; Oborník et al., 2015). Five basic cell stages (the coccoid cell, the autospore, the cyst, the zoosporangium and the zoospore) can be distinguished. The coccoid vegetative cell undergoes binary divisions and occasionally yields the autosporangium, a cluster of two, three or four daughter cells surrounded and protected by a shared thin membrane. Autospores are released from autosporangium and continue living as vegetative cells or, in adverse conditions, transform into cysts and deploy fatty acids in the cell wall making it thicker and more rigid. In favorable conditions, the cysts revert to coccoid stage and increase in size. In these enlarged cells, the organellar content is fragmented and transported to the cellular periphery as the zoosporangium is being formed; from this peripheral material zoospores are created. Up to eight zoospores were observed in the zoosporangium. The excystation of zoospores is rapid and takes approximately 2 min (Oborník et al., 2015). Then the zoospores discard their flagella and transform into encysted zoospores with a thick cell wall. Vegetative cells are formed from encysted zoospores completing the life cycle (Oborník et al., 2011). The zoospores might enable spreading into the more favorable conditions or finding a novel symbiotic partner (Oborník et al., 2015).

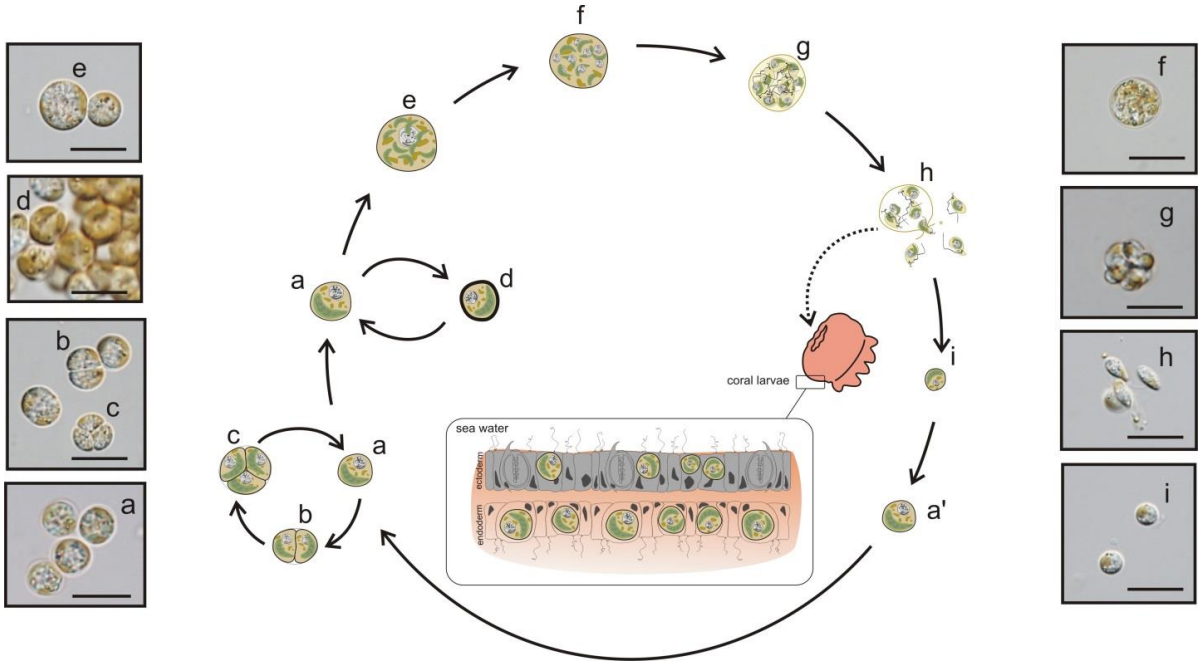

Fig.3: The diagram of *C. velia* life cycle with respective micrographs. The vegetative cell (a) divides into two (b), three (c), or four cells surrounded by thin membrane forming the autosporangium. The autospores (a) are released from autosporangium and transform into the cyst (d) or start to grow (e). The entire inner organellar content is fragmented (f), separated, and transported to the cell periphery forming the zoosporangium with the zoospores (g). The zoospores exscyst (h). The zoospores transform into encysted zoospores (i) and then into a new vegetative cell (a´). The flagellated zoospores might alternatively find the symbiotic partner (coral larvae) and start the symbiotic relationship. (Oborník et al., 2011; Oborník et al., 2015).

1.3.3. Ploidy and life cycle in Dinoflagellates

The majority of species in phylum Dinoflagellata are hetero- and autotrophic but about 140 species is strictly parasitic (Taylor, 1987; Noga and Levy, 1995; Nuismer and Otto, 2004). A similar variation as in trophic strategies can be seen in their life cycles. In general, an alternation of sexual and asexual reproductions is found, though sexuality has been proven only in a minor fraction of species. The life cycle comprises of five cell stages – the vegetative cell, the gametes, the planozygote, the hypnozygote and the planomeiocyte. The binary division of haploidic vegetative cell occurs in open water. Alternatively, haploidic gametes form and subsequently fuse into a diploid planozygote, guided by chemical attractants. The planozygote may undergo meiotic division into two haploid vegetative cells, or, in adverse conditions, it sinks to the bottom where it transforms into the diploidic dormant hypnozygote. Under favorable conditions, haploid vegetative cells are released from

the planomeiocyte in open water (Walker, 1984; Spector, 1984; Nagai, 2004; Figueroa et al., 2007; Dassow and Montresor, 2011).

1.3. The complexity of the genome and the number of chromosomes in Myzozoa

1.3.1. The complexity of the genome and the number of chromosomes in Apicomplexa

The evolution of Apicomplexa has been largely formed by an ancient event that occurred before the divergence of Myzozoa. Secondary endosymbiosis, the engulfment of a photosynthetic alga by a non-photosynthetic host eukaryote, led to major genetic and biochemical rearrangements inside the resulting organism (Gould et al., 2008; Keeling, 2013). Characteristic for the members of Apicomplexa are the single strongly reduced (in terms of genome) mitochondrion and the apicoplast with 35-kb sized circular genome, derived from the algal endosymbiont through gradual loss of photosynthetis capabilities (McFadden et al., 1996; Kohler et al., 1997). The reduced genomic size and function of these organelles are caused by gene loss or transfer of genetic information to the nucleus. This genetic transfer indeed necessitates a retargeting of the encoded protein repertoires back to the organelle where they function. The process strongly modified the nuclear genome composition, resulting in an evolutionary mosaic containing genes from both merging eukaryotes, the mitochondrion and the plastid (Archibald, 2009). These changes on the genetic level are hypothesized to have restricted Apicomplexa to parasitism through increasing dependence on metabolic supplies from outside (Wilson et al., 1996; Martin, 1998). The genome size ranges from 8.2 to 63 Mb contained on 4 to 14 chromosomes (for *Babesia bovis* and *Toxoplasma gondii*, respectively). In comparison, the genome of *Plasmodium falciparum* is 22.8 Mb on 14 chromosomes (Brayton et al. 2007; Dalmasso et al., 2014).

1.3.2. The complexity of the genome and the number of chromosomes in *Chromera velia*

The genome of *C. velia* contains wide range of genes, which were redirected to functions related to parasitism in Apicomplexa. For example, the orthologs of apicomplexan invasion-related motility genes were co-regulated with encoding genes of flagellar apparatus (Woo et al., 2015). The plastid of *C. velia* has a large genome (120 kb) with similarly low number of protein-coding genes (80) when compared to average apicoplast genomes. Consistently with

their relatedness, both *C. velia* and Coccidia (from the phylum Apicomplexa) use codon UGA to encode the amino acid tryptophan in the *PsbA* gene (Moore et al., 2008; Ralph et al., 2004). The mitochondrial genome of *C. velia* is even smaller than its apicomplexan and dinoflagellate counterparts, with only two protein-coding genes (*cox1* and *cox3*) and fragmented mito-rRNAs (Flegontov et al., 2015). The total size of *C. velia* nuclear genome is currently estimated to 193 Mb (Woo et al. 2015), while the number of chromosomes has not been determined.

1.3.3. The complexity of the genome and the number of chromosomes in dinoflagellates

The nuclear genome evolution of dinoflagellates was fundamentally influenced by lateral transfer of individual genes and massive gene transfer upon endosymbiosis. Dinoflagellates are known to possess some of the largest genomes recorded among eukaryotes, with size ranging from 1 500 to 245 000 Mb (Hackett et al., 2004; Wisecaver and Hackett, 2011), as supported by the DNA content ranging from 3 to 250 pg per cell (Spector, 1984). In some cases, this huge amount of nuclear DNA is contained in up to 200 chromosomes (Hackett et al., 2004). Unlike the nuclear genome, mitochondrial and plastid genomes of dinoflagellates (similarly to apicomplexans) are dramatically reduced, currently thought to possess the lowest gene content among known eukaryotic organelles. Only three protein-coding genes (*cox1*, *cox3*, and *cytb*) are found in the mitochondrial genome of dinoflagellates (Lukeš et al., 2009; Wisecaver and Hackett, 2011), while plastid genome is fragmented to several individual DNA molecules referred to as minicircles (Green, 2004).

1.4. Examination of ploidy and number of chromosomes by fluorescence *in situ* hybridization

1.4.1. Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) is a robust cytogenetic technique developed in 1980 (Bauman et al., 1980) used for detection of DNA and RNA sequences in cells and tissues. Both structural and numerical chromosomal changes can be detected, as well as specific DNA sequences within the interphase or metaphase nuclei. The fluorescence-labeled DNA probes are used for this detection, resulting in colored signals that can be identified using a fluorescence microscope (Levsky et al., 2003; Hu et al., 2014). In case of direct FISH, the signal can be observed directly after hybridization of the fluorophore-labeled

probe with the target sequence. In case of indirect FISH, the signal can be visualized after application of fluorophore-labeled antibody against hapten-labeled probe hybridized to the sequence of interest (Chen and Chen, 2013). During the years, this method has been continuously developed and optimized, resulting in a wide variety of applications and a high sensitivity (Levsky et al., 2003; Hu et al., 2014).

Tyramide signal amplification – fluorescence *in situ* hybridization (TSA-FISH) can be considered as an improvement of FISH enabling a high sensitivity detection of targets with low expression level that cannot be observed by standard direct or indirect FISH (Schriml et al., 1999). TSA is an enzyme-mediated method using horseradish peroxidase (HRP) and organic compounds, haptens (e.g. dinitrophenol (DNP) and digoxigenin (DIG) as conjugates), to generate high-density labeling of the target sequence of interest. After the hybridization of a hapten-labeled probe with the target sequence, HRP is introduced as an anti-hapten antibody conjugate. Thereafter, the solution of fluorophore-labeled tyramide is added, and tyramides are converted by HRP to highly reactive free radicals that bond covalently to tyrosine residues surrounding the HRP site. This results in a deposition of many more additional fluorophore molecules at the HRP binding site and rapid amplification of the resultant signal (Adams, 1992; Bobrow et al., 1992; Shindler and Roth, 1996).

1.4.2. Examination of ploidy and chromosome number by fluorescence *in situ* hybridization

FISH can be used to determine ploidy of an organism, if the probes are designed against genes that have only one physical copy in the genome, i.e. single copy genes (SC genes). Although each of these genes are represented only once in the genome of an organism, in total they are thought to contain most of the mRNA-encoding DNA, making them essential component of the genome (Lodish et al., 2000). The significant benefit of the use of FISH with SC gene probes in examination of ploidy is that the resulting signals are easily interpretable. A successful hybridization of the probe with SC gene on interphase (non-dividing) chromosome results in one positive fluorescent signal in a haploidic organism, while two signals are expected in a diploidic organism. This method is also employed in examination of polyploidy (Benkhalifa et al., 1993; Lengauer et al., 1997).

As well, FISH can be employed when inquiring the total count of chromosomes in an organism. In this case, the probes are designed against genuine chromosomal structures such

as the telomere or the centromere. Telomeres are long, repetitive and remarkably conserved sequences located on both ends of chromosome where they co-form the nucleoprotein caps that protect chromosomes and prevent the disruption of genetic information (Blackburn, 1991; O'sullivan and Karlseder, 2010). The length of telomeres is constantly being reduced because of the negative influence of environmental and genetic factors. Because of that, telomeres have to be regularly restored by the reverse-transcription activity of the enzyme telomerase (Sahin and Depinho, 2010; Romano et al., 2013). The telomere sequences (monomers) vary among animal and plant genomes, although *C. velia* and *P. falciparum* share the telomere sequence TTTAGGG typical for plants (Vernick et al., 1988; Bottius et al., 1998; Fulnečková et al. 2013). During evolution, telomere sequences can alter subtly but remain noticeably more conserved than centromeres that are completely missing in some organisms (Lysak, 2014 and Tran et al., 2015). Hence, telomere sequences are more suitable for probe targeting when performing FISH. A successful hybridization results in two visible signals per chromosome.

Despite the availability of nuclear genomic sequence, the questions of ploidy and chromosome number of *C. velia* remained unanswered. This work presents data that address these issues using these hybridization methods.

2. Aims of the thesis

- To determine ploidy of the alveolate alga *Chromera velia* by the use of single-copy-gene-targeted probes.
- To examine the total number of chromosomes in *C. velia* with the utilization of telomere-targeted probe.
- To employ tyramide signal amplification – fluorescence *in situ* hybridization as a principal technique in these chromosome-number studies.
- To utilize Southern blot and dot blot as methods to verify TSA-FISH results.
- To isolate nuclei from interphase *C. velia* cells to facilitate TSA-FISH employment.

3. Methods

3.1. *Chromera velia* cultivation

Solutions and reagents:

- F2 medium: 66.6g of Marine Reef Salt (Sera, Heinsberg, Germany) and 40 ml of Guillard's (F/2) Marine Water Enrichment Solution (Sigma) dissolved in 2 L of deionized H_2O, filter-sterilized)

C. velia strain CCMP2878 was used in our experiment. This strain was collected in Sydney Harbor, New South Wales, Australia, Pacific Ocean and was obtained from Bigelow, NCMA (The National Centre for Marine Algae).

The cultures were grown in F2 media at 26 °C under 12/12 h light/dark cycle and 17 μmol PAR/m^2/s. Cultures were harvested upon reaching the saturation point, characteristic by depletion of nutrients in medium, accumulation of storage lipids, arrest of cell division and entering the interphase. One-to-hundred dilution of a saturated culture in fresh medium was used as a starter culture for harvesting *C. velia* for all methods, taking app. 600 hrs to reach saturation.

3.2. Fluorescence *in situ* hybridization

3.2.1. Preparation of probes

3.2.1.1 Virtual design of single copy gene probes

In total, four probes were designed using Geneious (Biomatters) – three for single-copy (SC) genes and one for telomere repetitive sequence (see Tab.1).

Tab.1: The probes utilized in hybridization techniques. Both synthase genes are involved in the pyrimidine metabolic pathway of *C. velia*. The sequence of Topoisomerase II was obtained from the genome of *Toxoplasma gondii.*

Probe type and name	**Probe target**
Single copy gene probe 1 – SC1	CDP-MEP, i.e. 2-C-methyl-D-erythritol 2,4-Cyclodiphosphate Synthase
Single copy gene probe 2 – SC2	MecPP synthase, i.e. 2-C-Methyl-D-Erythritol-2,4-Cyclodiphosphate Synthase
Single copy gene probe 3 – SC3	Topoisomerase II
Telomere probe -TELO	TTTAGGG

Each probe for SC gene was designed in the length of 1050 bp with the preference to exon sequences, which contain repetitive sequences less likely, with an overlap to intron if necessary. Primers for these sequences (see Tab.2) were designed in Geneious. All probe and primer sequences were tested for uniqueness using Geneious (blasted against *C. velia* genome). The probes were also tested for tandem repeats content using Tandem Repeats Finder (Benson, 1999). Nucleotide composition analysis was performed with the Genom GC Calculator (Science Buddies).

Tab.2: The sequences of the probe cloning primers. The data was gained from Tm Calculator (BioLabs). FW – forward primer, RV – reverse primer.

Primer name	**Primer sequence**	**Length (b)**	**GC content (%)**	**T_m (°C)**
SC1 FW	GAGACGGCATTGTTCAAC	18	50	59
SC1 RV	GCAACAAATTTCTTGAAGGC	20	40	58
SC2 FW	GTTTCGAACCAGGTTTCTAT	20	40	57
SC2 RV	TGAAAAGGTTGACTCCGTAG	20	45	59
SC3 FW	CTTTCCCATGCTTTTGTCC	19	47	59
SC3 RV	ACACCCAAACATGGATTGG	19	47	61
TELO FW	$(TTTAGGG)^4$	28	43	66
TELO RV	$(CCCTAAA)^4$	28	43	66

3.2.1.2. Experimental synthesis of single-copy gene probes

Solutions and reagents:

- Selective LB agar: LB medium, 2% agar, 100 μg/ml ampicillin, 350μM isopropyl β-D-1-thiogalactopyranosid, 35 μg/ml X-gal

SC gene probes were amplified by PCR with Q5 Hot Start High-Fidelity DNA polymerase (NEB) according to the manufacturer`s protocol. Two-hundred ng of gDNA was used in every reaction. A reaction without template DNA served as a negative control. PCR was performed in MJ Mini thermal cycler (BioRad) under following conditions: initial denaturation (98 °C, 30 s), 30 cycles of denaturation (98 °C, 10 s), annealing (60 °C, 30 s) and extension (72 °C, 30 s), and final extension (72 °C , 2 min). The resulting products were purified with QIAquick PCR Purification Kit (Qiagen) and their quality was assessed using the NanoDrop 1000 spectrophotometer (Thermo Scientific) and agarose gel electrophoresis.

For cloning into the pGEM vector (pGEM®-T Easy Vector System, Promega), 3'-dATP overhangs were added to the blunt ends of the PCR products. Five µl of the each sample was incubated with 5 µl of PPP master mix (Top-Bio, Prague, Czech Republic) in water bath (72 °C, 30 min). Then, 2 µl of this reaction was mixed with 20 ng of pGEM vector and 5 U of T4 DNA ligase in 5-µl reactions and incubated at 12 °C, overnight.

With the resulting SC-pGEM vectors, TOP10 chemicompetent cells (Invitrogen) were transformed according to the manufacturer`s protocol. One hundred µL of transformed cell culture was applied on selective LB agar and incubated overnight at 37 °C. Nine white (positive) colonies were cultured in LB medium with 100 µg/ml of ampicilin. Plasmid DNA was then isolated using QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer`s protocol. Insert identity was tested by Sanger sequencing at GATC Biotech AG (Cologne, Germany) using M13 universal primers.

Probes were consequently amplified from positive plasmid DNA with the gene specific primers using Q5 High-Fidelity DNA Polymerase (NEB) according to the manufacturer`s protocol. One ng of plasmid DNA was used in every reaction. After the synthesis, the probes were purified by ethanol precipitation with sodium acetate (for details see Genomic DNA isolation, chapter 3.3).

3.2.1.3. Synthesis of telomere probe

The telomere probe was amplified by non-template PCR according to the protocol of Petr Nguyen (Laboratory of Molecular Cytogenetics, Institute of Entomology, Biology Centre CAS). Instead of target DNA, forward $(TTTAGGG)_4$ and reverse $(CCCTAAA)_4$ primers for telomere sequence of *C. velia* served as template. Five µl of each 10mM primer was mixed with 50 µl of the PPP Master Mix and 40 µl of nuclease-free water. This mixture was aliquoted into four 25-µl reactions to facilitate high thermal dynamics of the reactions. The non-template PCR reaction was then performed under following conditions: started with initial denaturation (94 °C, 90 s), followed by 29 cycles of denaturation (94 °C, 45 s), annealing (60 °C, 30 s), and elongation (72 °C, 60 s) and ended with final elongation (72 °C, 10 min). After the synthesis, the probe was purified by ethanol precipitation with sodium acetate (for details see Genomic DNA isolation, chapter 3.3.) and the quality of synthesis was verified by NanoDrop 1000 spectrophotometer and gel electrophoresis.

3.2.1.4. Labeling of the probes

All probes were labeled with Dinitrophenol-11-2'-deoxyuridine 5'-triphosphate i.e. DNP-dUTP (Perkin Elmer) by the means of nick translation (Nick Translation Kit, Abbott Molecular). Five hundred ng of each unlabeled probe DNA were mixed with 1.25 µl of 0.2mM DNP-dUTP, 2.5 µl of 0.1mM dNTP mix, 2.5 µl of 10×Nick Translation Buffer, 5 µl of Nick Translation Enzyme and adjusted to 25 µl with nuclease-free water. These reactions were then transferred into the MJ Mini thermal cycler (BioRad) and let incubate under the following conditions: lid 30 °C, elongation (15 °C, 75 min for SC probes, 15 °C, 90 min for telomere probe), denaturation (70 °C, 10 min). The samples were then cooled down on ice and purified immediately.

The labeled probes were purified using lab-made Sephadex columns. For each sample, 0.025g of Sephadex G-50 (Sigma) was blended with 400 µl of MilliQ ultrapure water and let hydrate (4 °C, 45 min). To create a column (see Fig.4), 150 µl of this mixture was transferred onto the filter of a 100-µl end-cut tip. The resin was settled by applying pressure from a pipette. In two similar steps, the rest of the Sephadex resin was applied to the column. These filled tips were then transferred into 1.5-ml tubes and centrifuged (1000 g, 1 min) to dehydrate the column. The columns were afterwards transferred into clean 0.5-ml tubes, which were set in the 1.5-ml tubes to allow centrifugation. Twenty five µl of labeled probe was transferred onto the Sephadex column and centrifuged (1000 g, 2 min). The size of the products was controlled by gel electrophoresis. The final size was about 500 bp for both SC and telomere probes.

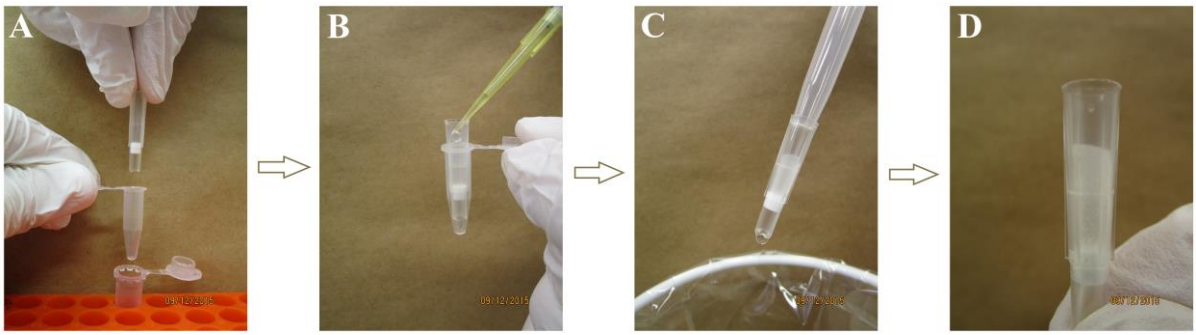

Fig.4: **A** – Set of 100-µl end-cut tip and 0.5-ml and 1.5-ml tubes. **B** – The loading of the Sephadex mixture, **C** – The resin settling by applying pressure from a pipette, **D** – The ready-to-use Sephadex column. The detailed description of the probe preparation is stated above.

3.2.2. Isolation of nuclei

Solutions and reagents:

- 1×PBS: 137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4, pH 7.4
- LLYC enzymatic solution: 1% Lysozyme (from hen egg, Roche), 1% CELLULYSIN Cellulase (from *Trichoderma viride*, Calbiochem), 1% Lysing Enzymes (from *Trichoderma harzianum*, Sigma) in 1×PBS

The nuclei were isolated from a fully grown culture (interphase chromosomes) with the use of the enzymatic and gentle mechanic methods. The culture was harvested on 0.2 μm filter using vacuum (100 mL of the culture per filter). The culture was then washed from the filter with 10 ml of 1×PBS solution and sedimented (6000 rpm, 5 min, 20 °C) in 15-ml tube. The supernatant was decanted and the cells were washed once more with 1×PBS using pipetting and vortexing. After centrifugation, the cells in pellet were resuspended in 10 ml of LLYC enzymatic solution and incubated overnight (30 °C, dark) while shaking. Next day, the sample was centrifuged and washed twice with 1×PBS. Finally, cells were fixed with freshly prepared 4% formaldehyde in PBS, vortexed rigorously (maximal intensity, 2 min) and stored at 4 °C for at least 3 h. This lysed fixed cell culture with extracted nuclei was then used in TSA-FISH method (for details see Tyramide signal amplification - Fluorescence *in situ* hybridization, chapter 3.2.3).

3.2.3. Tyramide signal amplification - Fluorescence *in situ* hybridization

Solutions and reagents:

- 1×PBS: 137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4, pH 7.4
- 1×TN Buffer: 0.1M Tris-HCl (pH 7.5) + 0.15M NaCl
- 1×TNB blocking buffer: 0.1M Tris-HCl pH 7.5, 0.15M NaCl and 0.5% Blocking reagent (PerkinElmer)
- 1×TNT Buffer: 1xTN + 0.05% Tween-20 (Sigma)
- 2×SSC: NaCl 300mM, 30mM trisodium citrate
- 20×SSC: NaCl 3M, 300mM trisodium citrate
- Hybridization mixture: 25 μl of deionized formamide, 5 μl of 20×SSC, 10 μl of 50% dextran sulfate, 30 ng of purified probe and sterile deionized water to the volume of 50 μl,(prepared freshly and kept on ice)

- Solution of DABCO and DAPI: 1.25 µl of DAPI stock (200µg/ml) in 500 µl of DABCO solution (2.33% DABCO (Aldrich), 20mM Tris-HCl pH 8.0 and glycerol)
- TSA solution: 2 µl of Fluorophore (Fluorescein) Tyramide Stock Solution in DMSO plus 98 µl of 1×Plus Amplification Diluent (Perkin Elmer)

Tyramide signal amplification - Fluorescence *in situ* hybridization (TSA FISH) was performed according to Paladino et al. (2014) with modifications according to Vladimír Krylov (Department of Cell Biology, Faculty of Science, Charles University in Prague, Czech Republic).

For each slide, 30 µl of fixed cell lysate (for details see Isolatin of nuclei, chapter 3.2.2) were first loaded on Superfrost microscope slides (Aldrich) and then spread and immobilized on surface using a heating plate (45 °C) and a tungsten needle.

Next, the slides were immersed into a Coplin jar with 1% hydrogen peroxide in 1×PBS (RT, 30 min, shaking) and washed three times with 1×PBS (5 min each round, RT, with agitation). After washing, each slide was incubated in humid chamber with 100 µg/ml of RNase A in 1×PBS under a cover glass (24×50mm) (37 °C, 1 h, paper towels soaked with 2×SSC). Then, the cover glasses were removed and the slides were washed again three times with 1×PBS (RT, 5 min each round, with agitation). The slides were then immersed in 5×Denhardt´s solution in H_2O (37 °C, 30 min, shaking). After removal from the Coplin jar, 50 µl of the hybridization mixture was loaded on each slide. The slides were covered with cover glass (24×50mm) and denaturated immediately in a Mastercycler thermal cycler with *In Situ* Block Adapter (Eppendorf) (70 °C, 5 min). After the denaturation, the slides were transferred into a wet chamber pre-warmed to 37 °C and hybridization took place in the incubator (37 °C, overnight 12-16 h)

Next day, the cover glasses were removed and the slides were washed three times in 50% formamide in 2×SSC (42 °C, 5 min each round, with agitation). After formamide washing, the slides were washed three times with 2×SSC (5 min each round, RT, with agitation) and then once with 1×TNT buffer (5 min, RT, with agitation). The superfluous liquid was then shaken off and wiped from the bottom of the slides. After placing into a wet chamber, 400 µl of the 1×TNB blocking buffer was applied on each slide. The wet chamber was sealed and the slides were incubated without cover glasses (30 min, RT). The blocking buffer was shaken off and the bottoms of the slides were wiped. Two hundred µl of primary antibody

(ANTI-DNP HRP conjugate) in 1×TNB (diluted 1:1000 for SC probes and 1:2000 for TELO probe) were then applied on the slides, followed by incubation (1 h, 37 °C) in a humid chamber under cover glasses (24×50 mm). Then, the slides were rinsed three times with 1×TNT (RT, 5 min each round, with agitation).

The following steps were performed in a darkroom. The redundant TNT was shaken off and 100 µl of the TSA solution was applied on each slide. Each slide was afterwards covered with cover glass (24×50mm). The slides were then incubated in humid chamber (RT, 10 min in the case of TELO probe and 15 min for SC probes, dark). The slides were then rinsed three times with 1×TNT with a dropper and then washed three more times in a Coplin jar (RT, 5 min each round, with agitation, dark). The slides were rinsed with 1% PhotoFlo in MilliQ ultrapure water (RT, 1 min, with agitation, dark) and excessive fluid was drained off. Finally, 25 µl of the DABCO and DAPI solution was applied and each slide was covered with cover glasses (24×40mm). Excessive fluid was squeezed out with a gentle pressure and the samples were sealed with nail varnish. The slides were stored at 4 °C in the dark before being examined by fluorescence and confocal microscopy.

3.2.4. Fluorescence and confocal microscopy

Slides were previewed on an Olympus BX53 fluorescence microscope equipped with a mercury arc lamp, operated by CellSense Dimension software (Olympus) for taking and processing of images. Confocal scanning was performed on an Olympus FluoView FV1000 microscope equipped with a multi-argon laser and a mercury arc lamp. The software FV10-ASW 3.0. (Olympus) was used for taking and processing of the images.

3.3. Genomic DNA isolation

Solutions and reagents:

- Lysis buffer: 500mM NaCl, 50mM EDTA, 100mM Tris-Cl pH 8.5

DNA extraction was conducted using a modified phenol-chloroform method. At one time, about 2 L of *C. velia* culture was used for the gDNA extraction with a yield of approximately 75 μg/1 L of culture.

Fully grown *C. velia.* culture was harvested through 0.2 μm filter using vacuum (500 ml of the culture per one filter). The cells were washed from the filter with 5 ml of 1×PBS, centrifuged (6000 rpm, 5 min, RT) and washed once more. After centrifugation and decanting of the supernatant, lysis buffer was added. The culture was then treated with ten freeze-thaw cycles in liquid nitrogen (30 s) and water bath (60 °C, 60 s). After this treatment, proteinase K was added to final concentration 100 μg/ml and the culture was incubated 3 h at 50 °C shaken gently every 30 min. Afterwards, RNase A was added to final concentration 10 μg/ml and the cells were treated 15 min at 37 °C.

Next, 1 v/v phenol solution (Sigma) was added, and the sample was mixed by shaking for 5 min at RT. The sample was then centrifuged (13000 rpm, 3 min, RT) and the aqueous upper layer was transferred into new clean tubes. Phenol extraction was repeated three times in total. To remove phenol, the sample was once treated with the phenol:chloroform:isoamylalcohol (25:24:1) and, thereafter, twice with chloroform:isoamylalcohol (24:1) using the same phase-separation procedure.

Finally, the sample was ethanol-washed. DNA was precipitated by addition of 2.33 v/v absolute ethanol and 0.1 v/v 3M sodium acetate (pH 8.0) and cooling at -80 °C for 1 h. Following centrifugation (13000 rpm, 15 min, 12 °C), the supernatant was removed. The pellet was then three times washed by addition of 200 μl of 70% cold ethanol (-20 °C) and centrifuging (13000 rpm, 5 min, RT). Finally, the supernatant was removed, the redundant ethanol was let evaporate, and the pellet was dissolved in 50 μl of nuclease-free water. The concentration and purity of the extracted DNA was examined by NanoDrop 1000 spectrophotometer and by agarose electrophoresis. DNA was stored at -20°C until further use.

Genomic DNA of *Pisum sativum* (PS) and *Cydia pomonella* (CP) were used as a control in Southern blot using DIG-labeled probes. PS gDNA was obtained from the Laboratory of Molecular Cytogenetics (Institute of Plant Molecular Biology Biology Centre AS CR, v.v.i.) and CP gDNA was acquired from the Laboratory of Molecular Cytogenetics (Institute of Entomology, Biology Centre AS CR, v.v.i.).

3.4. Dot Blot

Solutions and reagents:

- Neutralization buffer: 0.5M Tris-Cl (pH 7.2) and 1M NaCl
- Hybridization phosphate buffer: 0.5M phosphate buffer pH 7.2, 1mM EDTA pH 8.0, 7% SDS, 1% bovine serum albumin

This method was employed to verify functionality and specificity of the radioactively labeled probes and the quality of gDNA extractions. Decimal serial dilutions of SC-pGEM vectors and TELO template (50 ng/µl, 5 ng/µl, 500 pg/µl and 50 pg/µl for SC-pGEM plasmids and 5 ng/µl, 500 pg/µl, 50 pg/µl and 5 pg/µl for TELO) were prepared and after denaturation (95 °C, 5 min, then chilled immediately on ice), 2 µl of each dilution was dripped onto the Amersham Hybond-N+ nylon membrane (GE Healthcare). In parallel, 1 µg of denatured gDNA was dripped onto the membrane. The drops were dried and the membranes were auto-cross-linked by UV Stratalinker 1800 (Stratagene). The membranes were then soaked in neutralization buffer (RT, 15 min) and directly prehybridized in phosphate buffer in the ProBlot hybridization oven (Labnet) (65 °C, 1 h, slow rotation) and then immediately hybridized in an identical solution containing the respective probe (65 °C, overnight, slow rotation). Washing and detection were performed as below (for details see Southern blot, chapter 3.5.).

3.5. Southern blot

The copy number of chosen genes was assessed using Southern blotting. Alpha-^{32}P-dATP- and DIG-dUTP-labeled probes were used in parallel and their performance was compared. The Southern blot using DIG-labeled probes was performed according to Traut et al. (2007) with modifications.

3.5.1. DNA digestion and DNA separation by gel electrophoresis

Solutions and reagents:

- TE buffer: 10mM TRIS-HCl and 1mM EDTA, pH 8.0
- 0.5×TBE: 110mM Tris-HCl, 90mM boric acid, 2.5mM EDTA, pH 8.3

C. velia genomic DNA (gDNA) was digested by combinations of restriction endonucleases (50 µl reactions, 6 µg of DNA per sample, 60 U of each enzyme per reaction, NEB HF enzymes). The mix of gDNA and specific endonucleases was digested at 37 °C, overnight. Next day, the digestion was stopped by heat inactivation (80 °C, 20 min). All utilized endonucleases were zero cutters, i.e. enzymes that do not digest the target DNA sequence of interest. The enzymes were selected to cut in the vicinity of the target sequence.

Selected restriction endonucleases:

EcoRI HF, EvoRV HF, NcoI HF, KpnI HF, SalI HF, PvuI HF

Protocol using radioactivity-labeled probes:

The full volume of the samples (50 µl) was mixed with appropriate volume of Thermo Scientifics 6× Loading Dye and loaded onto a 0.7% agarose gel buffered with 0.5×TBE. The DNA fragments were then separated (4 V/cm). GeneRuler 1 kb DNA Ladder (ThermoFisher) was used as a marker. After the separation, the gel was stained with GelRed (Biotium) (RT, 45 min, with gently agitation) and briefly visualized on UV transilluminator to confirm presence and appropriate distribution of DNA.

Protocol using DIG-labeled probes:

The full volume of the samples (30 µl) was mixed with appropriate volume of Thermo Scientifics 6× Loading Dye and loaded onto a 1% agarose gel in 1×TBE. The DNA fragments were then separated (4 V/cm). The DIG marker III (Roche) was used as a marker. After the separation, the gel was stained with ethidium bromide (Sigma) (RT, 5 min, with gently agitation) and briefly visualized on UV transilluminator to confirm presence and appropriate distribution of DNA.

3.5.2. Blotting

Protocol using radioactivity-labeled probes:

Solutions and reagents:

- Alkaline transfer buffer: 0.4N NaOH and 1M NaCl
- Neutralization buffer: 0.5M Tris-Cl (pH 7.2) and 1M NaCl

The gel was transferred onto a glass plate and the unnecessary parts of the gel were cut off. The gel was then treated in 0.2N HCl (RT, 15 min, with gentle agitation), followed by soaking in alkaline transfer buffer (two times, RT, 15 and 20 min, with gentle agitation). Meanwhile, the Amersham Hybond-N+ nylon membrane (GE Heathcare) was firstly briefly soaked in deionized water and transferred into alkaline transfer buffer (RT, 5 min). After this pretreatment, the blotting apparatus was assembled and the DNA samples were transferred onto the membrane in alkaline buffer (RT, 20 h). Next, the membrane was washed in neutralization buffer (RT, 15 min). The DNA was bound to the membrane by UV Stratalinker 1800 (Stratagene).

Protocol using DIG-labeled probes:

Solutions and reagents:

- Denaturation solution: 0.5M NaOH, 1.5M NaCl
- Neutralization solution: 0.5M Tris-HCl, 3M NaCl, pH 7.5
- 20×SSC: 3M NaCl, 0.3M sodium citrate, pH 7.0
- 6×SSC: 0.9M NaCl, 0.09M sodium citrate, pH 7.0
- 2×SSC: 0.3M NaCl, 0.03M sodium citrate, pH 7.0

The gel was treated in 0.25M HCl (RT, 10 min, with gentle agitation), washed two times with distilled water and soaked into the Denaturation solution (RT, 2 × 15 min, with gentle agitation). The gel was then again washed two times with distilled water and soaked into the Neutralization solution (RT, 2 × 15 min, with gentle agitation). Finally, the gel was prepared and the DNA fragments were transferred onto the membrane in 20×SSC (RT, 20 h). After the blotting, the membrane was washed in 6×SSC (RT, 5 min). The DNA was fixed to the membrane by Stratalinker 1800 (Stratagene). The membrane was then briefly washed in 2×SSC.

3.5.3. Probe labeling and hybridization

Protocol using radioactively-labeled probes:

Solutions and reagents:

- Hybridization buffer: same as prehybridization buffer, with labelled probe added
- Prehybridization buffer: 0.5M phosphate buffer pH 7.2, 1mM EDTA, 7% SDS, 1% bovine serum albumin

The probes were labeled with [α-32P]-dATP (M.G.P.) using random priming (DecaLabel DNA Labeling Kit, Fermentas). For each sample, 100 ng of unlabeled probe DNA was mixed with 5 µl of Decanucleotide in 5×Reaction Buffer and filled to 40 µl with nuclease free water. The DNA was denatured in boiling water bath for 8 min and snap cooled on ice. Next, 6 µl of mixture consisting of 3 µl of Mix A, 2 µl of [α-^{32}P]-dATP and 1 µl of Klenow Fragment (exo–, 5 U/µl) were added and the tubes were incubated in water bath (37 °C, 5 min). Afterwards, 4 µl of dNTP Mix was added and the incubation continued for another 5 min (37 °C). The labeling reaction was then stopped by addition of 1 µl of 0.5M EDTA, pH 8.0. The labeled DNA probes were then purified from incorporated nucleotides using a Sephadex column (for details see Labeling of the probes, chapter 3.2.1.4.). Purified radioactively labeled probes were then immediately used.

Before hybridization, the cross-linked membrane was cut to 4 pieces corresponding with the four respective genes (SC 1-3 and TELO genes), each piece transferred into a 50-ml falcon tube (for hybridization inserted into roller bottles) and treated with the prehybridization buffer inside a prewarmed ProBlot hybridization oven (Labnet) (65 °C, 2 h, slow rotation). The membranes were then transferred into glass roller bottles, and 10 mL fresh prehybridization buffer plus corresponding denatured probe were added to each membrane. Hybridization took place in hybridization oven (65 °C, 48 h, slow rotation).

Protocol using DIG-labeled probes:

The probes were labeled with digoxigenin (DIG) (PerkinEmer) using nick translation (Nick Translation Kit, PerkinEmer). For one reaction, 500 ng of unlabeled probe DNA was mixed with 2.5 µl of 10×Translation Buffer, 2.5 µl of 10×dNTP Mix for dUTP labeling, 5 µl of Nick Translation Enzyme Mix and filled to 25 µl with nuclease-free water. The template DNA was then labeled in thermocycler (15 °C, 75 min). The reaction was stopped by heat

shock (70°C, 10 min, then chilled on ice). The labeled DNA probes were then purified from incorporated nucleotides with Sephadex column (for details see Labeling of the probes, chapter 3.2.1.4.). Purified DIG-labeled probes were then immediately used.

The membrane was initially prehybridized in 18 ml of pre-warmed DIG Easy Hyb (Roche) (42 °C, 60 min, with gentle agitation) and then hybridized in 10 ml of pre-warmed DIG Easy Hyb with the denatured DIG-labeled probe (42 °C, overnight, with gentle agitation).

3.5.4. Washing of the membrane and signal detection

Protocol using radioactively-labeled probes:

Solutions and reagents:

- Phosphate SDS Washing Solution 1: 40mM phosphate buffer pH 7.2, 1mM EDTA, 5% SDS, 0.5% bovine serum albumin
- Phosphate SDS Washing Solution 2: 40mM phosphate buffer pH 7.2, 1mM, 1% SDS

After the hybridization, the membrane was washed twice (65 °C, 5 min, with rotation) with Phosphate SDS Washing Solution 1. After the first washing, Phosphate SDS Washing Solution 2 was used for additional rinsing. Second step of washing was conducted six times in total and the signal-to-noise ratio was continuously examined with a Geiger counter (Model 3 Survey Meter, Ludlum). The used rinsing solutions were collected and disposed according to effective radioactive waste handling precautions.

Pieces of the membrane were then dried on a paper towel and deposited side-by-side between two sheets of plastic foil. Wrapped membrane was stored in an Exposure Cassette (GE Healthcare) exposed to BAS Storage Phosphor Screen (GE Healthcare) for 36 h. The signals were then visualized on Variable Mode Imager Typhoon 9410 (Amersham, Biosciences).

Protocol using DIG-labeled probes:

Solutions and reagents:

- 1×Blocking solution: 1×TBS, 5% fat free dry milk in
- 1×Washing buffer: 1×TBS, 0.3% Tween
- Stringent wash buffer I: 2×SSC, 0.1% SDS

- Stringent wash buffer II: 0.2×SSC, 0.1% SDS
- 1×Detection buffer: 0.1M Tris-HCL, 1M NaCL

All washing and incubation steps were performed with gentle agitation. The membrane was washed with sufficient volume of Stringent wash buffer I (RT, 2×5 min) followed with Stringent wash buffer II (68 °C, firstly 15 mint, then 20 min). Afterwards, the membrane was rinsed with 1×Washing buffer (RT, 5 min) and then incubated in 1×Blocking solution (RT, 45 min). After the blocking, the membrane was incubated in Anti-DIG-AP solution (Roche) (RT, 1 h). Finally, the excess ANTI-DIG-AP conjugates were washed two times with 1×Washing buffer (RT, 15 min).

Afterwards, the membrane was incubated in 1×Detection buffer (RT, 5 min). The membrane was briefly dried on a blotting paper and then transferred on the plastic film. Approximately 3 ml of CDP-Star Chemiluminescent Substrate (Roche) was dropped onto the membrane, which was immediately covered with the second layer of a plastic film and the membrane was incubated 5 min at RT (without agitation). The excess Chemiluminescent Substrate was disposed and the membrane was heat-sealed in a plastic film. The result of the Southern hybridization was then visualized by the charge-coupled device camera LAS-3000 (Fujifilm).

4. Results

4.1. Isolation of nuclei

The isolation of nuclei proved to be fundamental for a successful performance of TSA-FISH technique. Without this isolation, the labeled specific probed cannot penetrate the thick cell wall of *C. velia* and hybridize with target sequences of interest in the nuclei (Fig.6). Before the FISH experiment, this finding was supported by DAPI staining, when the released nuclei were, in comparison with the cell-located nuclei, multiple stained (see Fig.5, 1a). This finding verified that cell wall of *C. velia* represents a poorly penetrable barrier even for a small fluorescent molecule such as DAPI.

The cell wall was digested by a mixture of enzymes (see Methods, isolation of nuclei, chapter 3.2.2., Solutions and reagents, LLYC enzymatic solution). As a result, the majority of cells lost partially or completely their cell wall and under light microscope appeared as protoplasts. These protoplasts were subsequently mechanically treated, which led to their lysis and release of nuclei. The success rate of this method was approximately fifty percent, i.e., we were able to isolate nuclei from around half of cells. With the nuclei, other structures were released and the plastid was severely reduced or completely lost (see Fig.4 1b and Fig.5 2b). In this chapter, the images documenting DAPI staining are intentionally overexposed to demonstrate not only the nuclei, but also other cellular structures and residues.

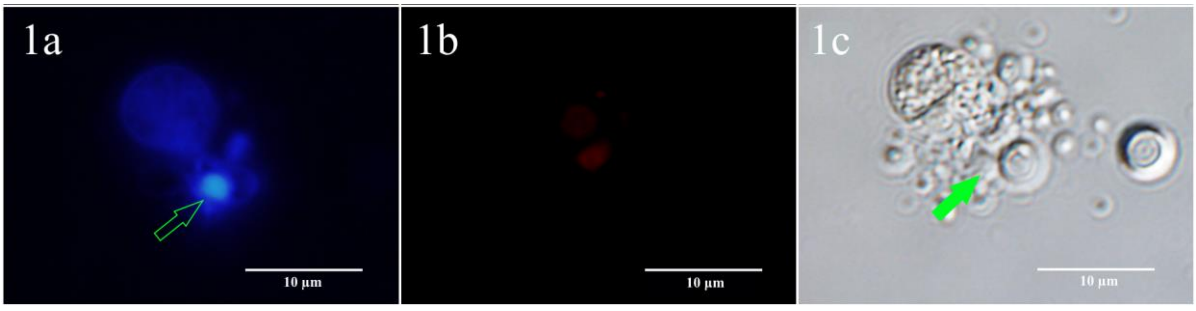

Fig.4: The fluorescence and light-microscopy image of lysed cell with released cell material. **1a** – Isolated DAPI-labeled nucleus. **1b** – small plastid residues (autofluorescence). **1c** – bright field image of the same exposition. Green arrows indicate the isolated nucleus. The images were captured by Olympus BX53 fluorescence microscope.

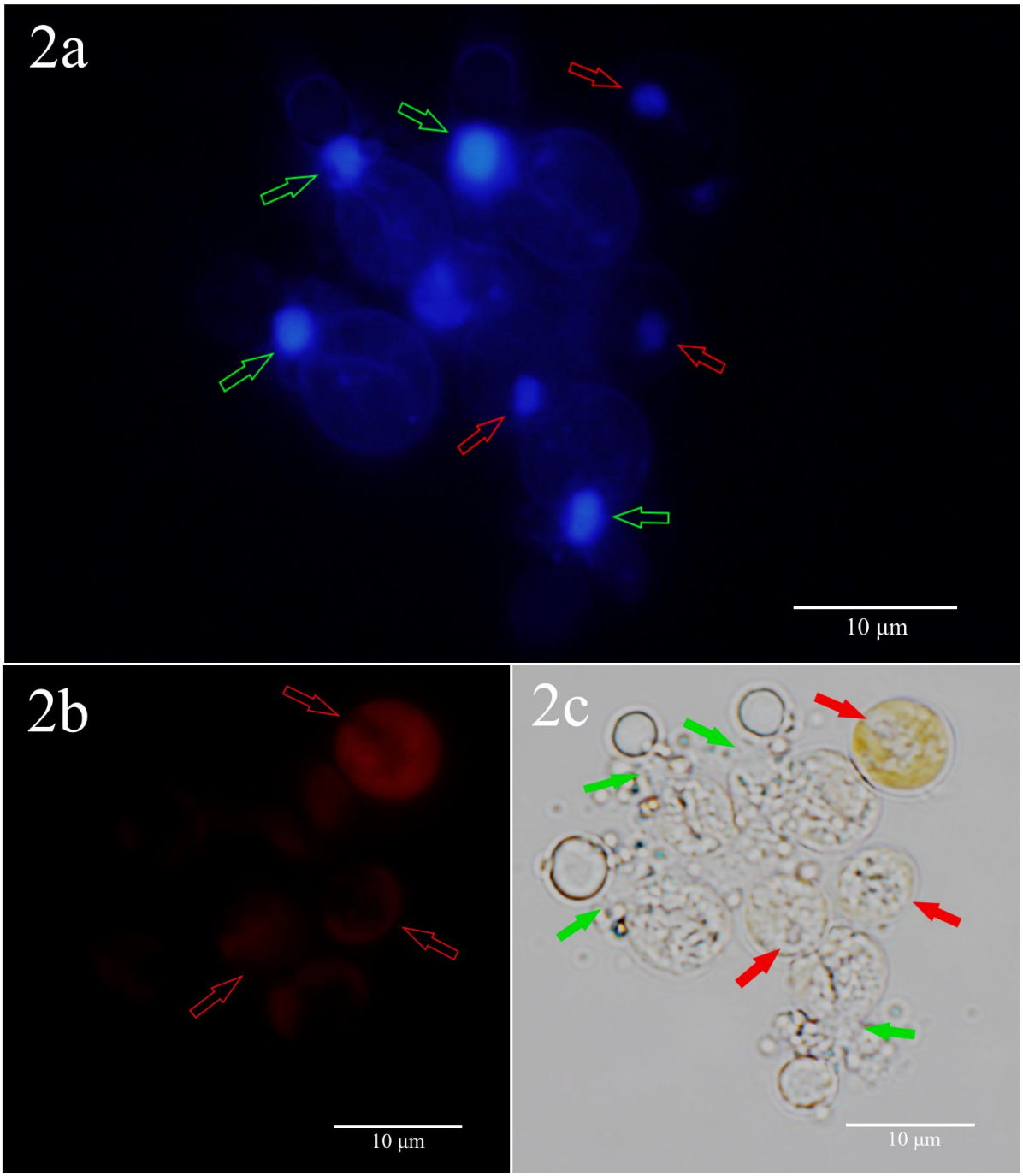

Fig.5: The fluorescence and light-microscopy images of *C. velia* cells after isolation of nuclei. **2a** – The fluorescence comparison of nuclei labeled by DAPI. The isolated nuclei (green arrows) are significantly better stained than nuclei located to intact cells (red arrows). **2b** – The comparison of plastid autofluorescence reduction in lysed and non-lysed cells. Red arrows indicate non-lysed cells with continual plastids. Plastids are absent or ruptured in lysed cells. **2c** – The arrows indicate the location of the nuclei. The plastids can be seen as brownish structures (compare with 2b) inside non-lysed cell – red arrows. The images were captured by Olympus BX53 fluorescence microscope.

4.2. Tyramide signal amplification – Fluorescence *in situ* hybridization

The ploidy and the total count of chromosomes in *C. velia* were inquired employing TSA-FISH. The resulting signals are visible as the green spots (fluorescein fluorescence). The Fig.6 demonstrates the test of *C. velia* cell wall permeability.

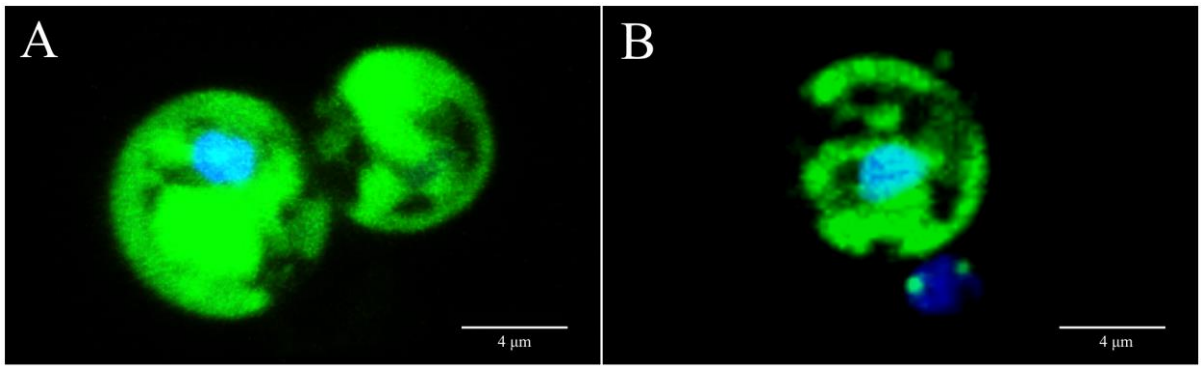

Fig.6: *C. velia* cell wall permeability for labeled DNA probes. **A** – Non-lysed cells, the probes cannot penetrate the cell wall and hybridize with the target sequences in nuclei. The resulting signal is dispersed on the cell surface. **B** – The comparison of unpenetrable non-lysed cell with – the signal is scattered on the cell surface and the isolated nuclei – the telomere probe is hybridized, resulting in two bright signals. The images were captured by Olympus FluoView FV1000 microscope.

4.2.1. The ploidy of *C. velia*

TSA-FISH was performed with probes for three different single copy genes (SC1 – CDP-MEP, SC2 – MecPP synthase and SC3 – Topoisomerase II). These probes were utilized in three biological replicates giving an identical result. Under microscope, there was only one fluorescent signal in all observed nucleus (see Fig.7 and Fig.8). These single signals indicate that *C. velia* possesses only one set of chromosomes, i.e. that *C. velia* is clearly a haploid organism.

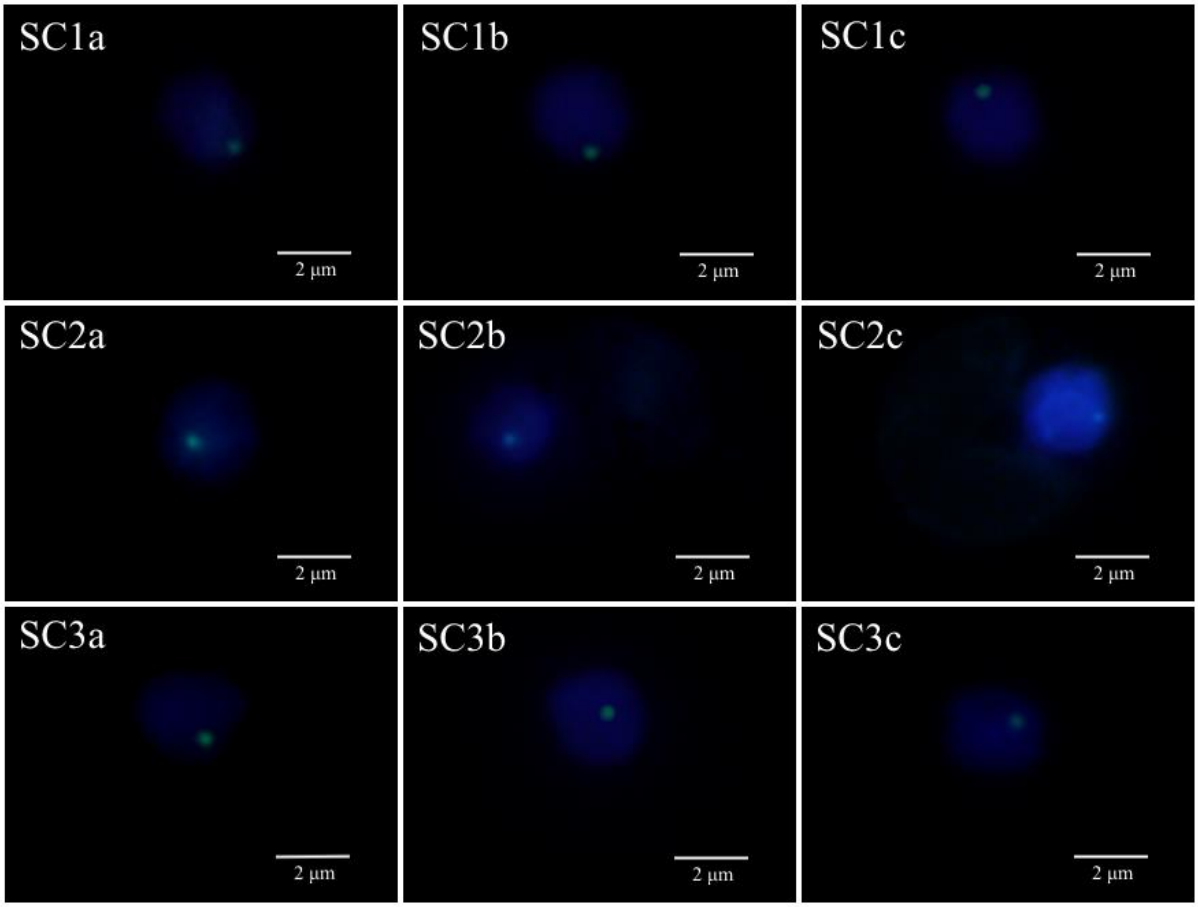

Fig.7: The result of TSA-FISH – the DAPI-labeled nuclei with fluorescent signals of SC probes. **SC1-3** correspond with a particular utilized probe, **a-c** represent three repetitions of the experiment. Only isolated nuclei are shown. **SC2b** and **SC2c** are occupied by the remnants of the lysed cells. The images were captured by Olympus BX53 fluorescence microscope.

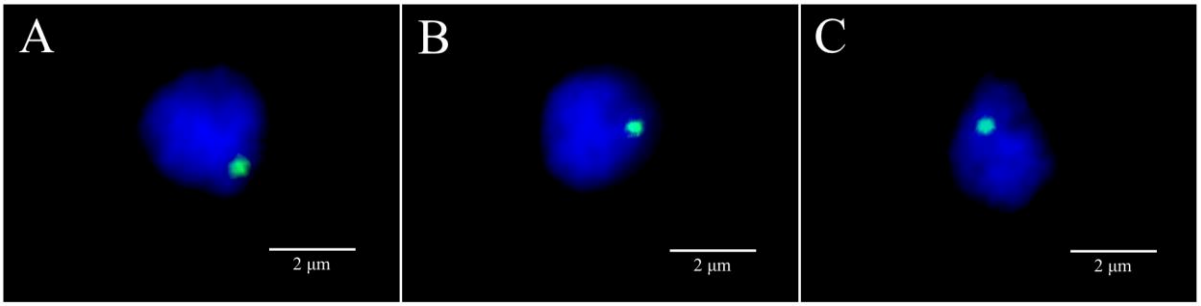

Fig.8: The result of TSA-FISH – the DAPI-labeled nuclei with fluorescent signals of SC probe. All images are from the last of TSA-FISH revision (**A**, **B** and **C**). The images were captured by Olympus FluoView FV1000 microscope and the signals were merged from several layers of a 3D scan.

4.2.2. The total number of chromosomes in *C. velia*

The probe for telomere sequence TTTAGGG was used to examine the total count of chromosomes in *C. velia*. Three biological repetitions of the experiment were conducted with the same result. When signals were visible, we always observed two signals (see Fig.9 and Fig.10). Based on the fact that every chromosome has two telomere sequences (one at each end), these two signals indicate that *C. velia* possesses only one chromosome in total.

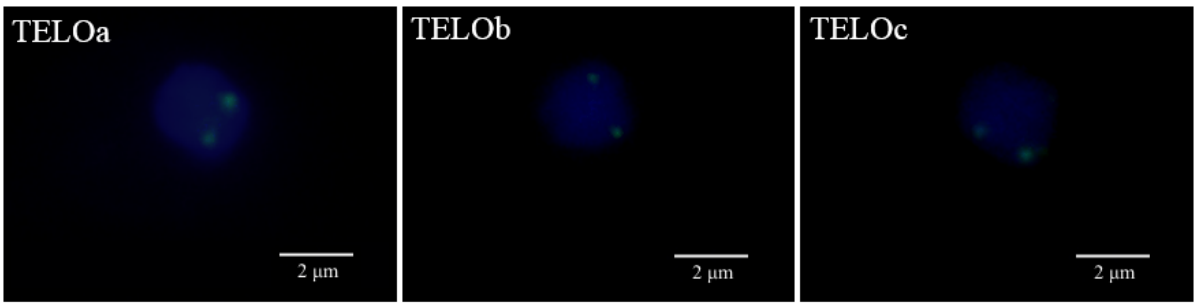

Fig.9: The result of TSA-FISH – the DAPI-labeled nuclei with fluorescent signals of TELO probe. The TSA-FISH experiment was repeated three times (a, b and c). All images were captured by Olympus FluoView FV1000. The images were captured by Olympus BX53 fluorescence microscope.

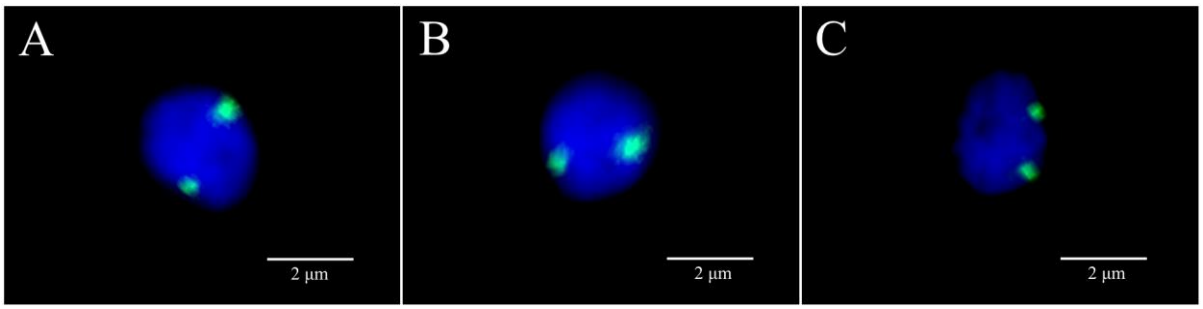

Fig.10: The result of TSA-FISH – the DAPI-labeled nuclei with fluorescent signals of TELO probe. All images are from the last TSA-FISH revision (**A**, **B** and **C**). The images were captured by Olympus FluoView FV1000 microscope and the signals were merged from several layers of a 3D scan.

4.3. Dot Blot

At first, dot blot technique was performed to verify the specific hybridization of the Alpha-^{32}P-dATP-labeled probes. The respective SC-pGEM plasmid (for SC probes) or the product of non-template PCR (for TELO probe) were dripped in serial dilutions onto nylon membranes. The telomere DNA (T DNA) template was used as a negative control for SC

probes, and vice versa, SC1-pGEM was used as negative control for the TELO probe. All probes hybridize specifically, i.e. apparent and decreasing signals for all positive controls and no signal for negative controls are visible (see Fig.11). The ability of probes to hybridize with *C. velia* genomic DNA was also assayed on equal amounts of two independent extractions (denominated a and b, Fig.11). All probes were able to hybridize with both gDNA extractions, although signal was better for extraction "a". This extraction was further used for Southern blotting.

Additionally, this experiment gave evident positive result for the TELO probe. TELO probe hybridized strongly, with multiple times greater intensity in comparison to the SC probes. This verified the presence of the TTTAGGG sequence in genomic DNA of *C. velia* and also the functionality of the TELO probe.

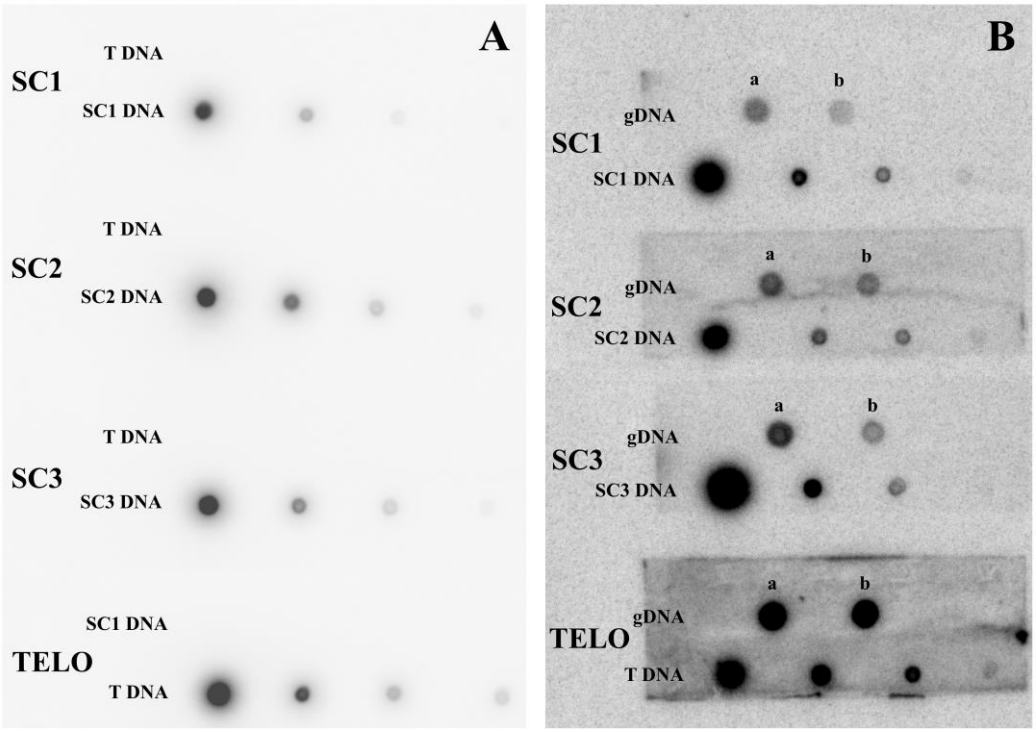

Fig.11: The results of the dot blot technique. **A** – The control of the probe specificity. SC DNA served as a positive and T DNA as a negative control of the SC probes (SC1-3) specificity. For the specificity test of telomere probe (TELO), the design of the experiment was reversed. **B** – the control of quality of two various genomic DNA extractions (gDNA a and b) and the ability of probes to hybridize with this gDNA extractions. The serial dilution of appropriate plasmid DNA (for SC1-3 probe) and telomere DNA (for TELO probe) served as a positive control.

4.4. Southern blot

The Southern blot technique was employed to verify the results of TSA-FISH. Two protocols were followed in Southern blot technique. Alpha-^{32}P-dATP- and DIG-dUTP-labeled probes were used in parallel, giving identical results. The genomic DNA of *C. velia* was digested with selected combinations of restricted endonucleases to produce kb-range fragments. For the estimated lengths of target DNA after the digestion and the used enzyme combinations, see Tab. 3. Before the blotting, the digested gDNA was separated by the gel electrophoresis and no clear bands were observed, in line with a random cutting pattern.

The ploidy of *C. velia* was examined by the hybridization of SC probe with the target single copy gene sequences, which were blotted onto the membrane after the specific digestion of *C. velia* gDNA (Fig.12). All SC probes hybridized with sequences of the estimated lengths (compare Fig.12 with Tab. 3.). For all SC genes, only one band appeared on the membrane. This result supports the finding of TSA-FISH that *C. velia* is a haploid organism.

Tab.3: The combinations of High Fidelity Restriction Endonucleases used for the digestion of genomic DNA of *C. velia*. The target product estimated lengths are shown in brackets. These lengths were calculated using Geneious (Biomatters).

Gene:	**1st combination**	**2nd combination**
SC 1	*NcoI* + *KpnI* (4,728)	*NcoI* + *EcoRV* (5,492)
SC 2	*PvuI* (3,776)	*SalI* + *EcoRV* (4,234)
SC 3	*EcoRI* (3,098)	*EcoRV* + *NcoI* (4,931)
TELO	*EcoRI* + *EcoRV*	*EcoRV* + *NcoI*

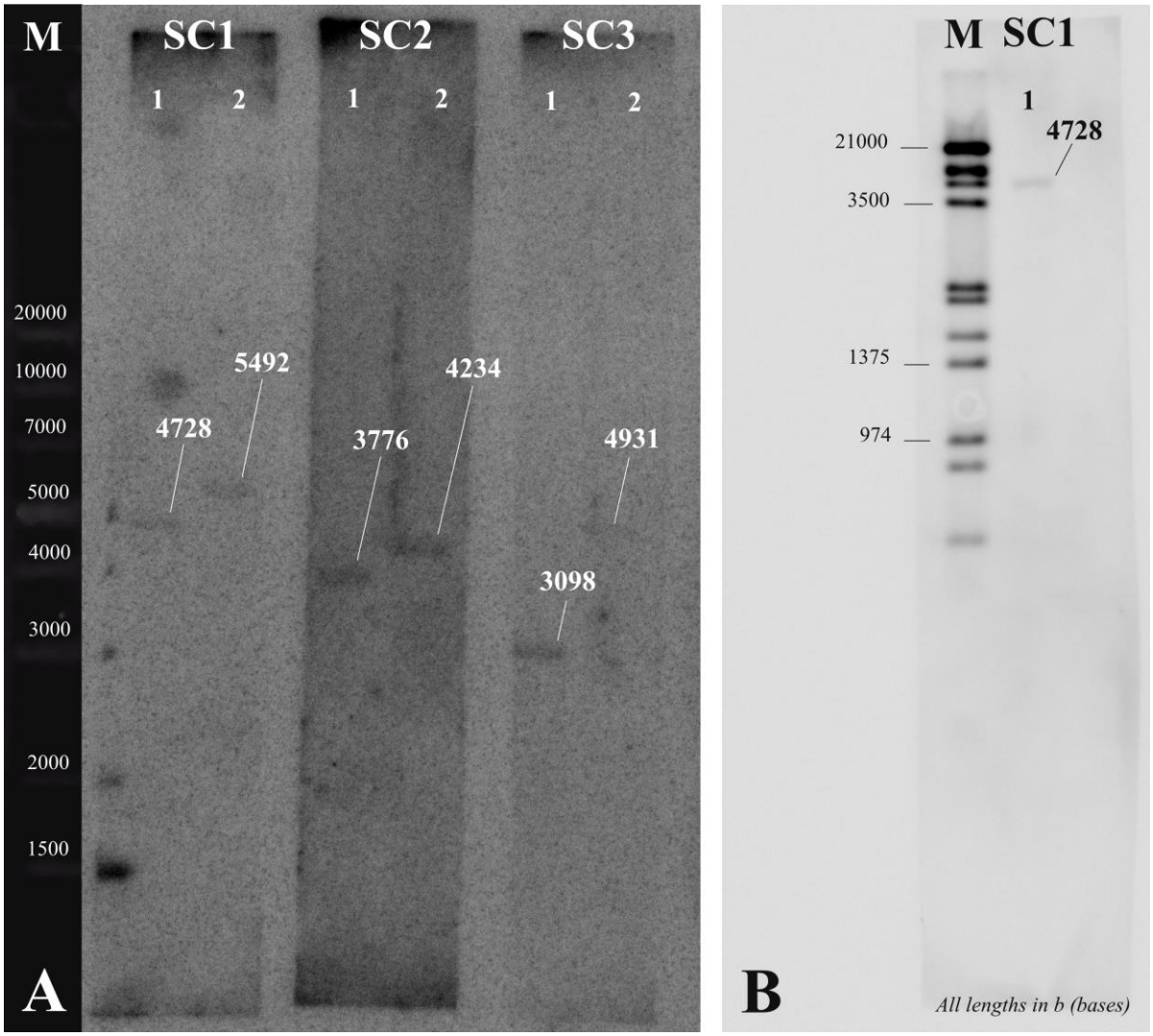

Fig.12: Southern blot using radioactive and DIG-labeled SC probes. **A** –The membrane using Alpha-^{32}P-dATP-labeled probes for all SC genes. For each SC gene, two combinations of restriction endonucleases were used for the digestion of gDNA (lanes 1 and 2). The exposition time was 36 h. **B** – The membrane using DIG-labeled SC1 probe (combination of enzymes 1). The time of exposition was 2 min. The estimated lengths of every hybridization signal (see Tab.3) and the size ladder (M) are shown.

The total chromosome count of *C. velia* was investigated by hybridization with TELO probe. The number of chromosomes was first examined by Southern blot using radioactively labeled telomere probe, but the hybridization was unsuccessful (see Fig.13, A). Hence, the Southern blot was repeated using a DIG-labeled probe. The genomic DNA of *C. velia, P. sativum and C. pomonella* were digested with two identical combinations of enzymes (see Tab.3). Genomic DNA of *P. sativum* (PS) having the same telomere sequence as *C. velia* (TTTAGGG) was used as a positive control, while gDNA of *Cydia pomonella* (CP) with the telomere sequence TTAGG was used as a negative control. The result of the Southern blot

using DIG-labeled probes was similar as for the radioactively labeled probe, i.e. there was no visible signal for *C. velia*. In contrast, we observed intense signal from the positive control and even weak signal from the negative control, which indicates unspecific binding of telomere probe to the telomere sequence of CP (see Fig.13, B).

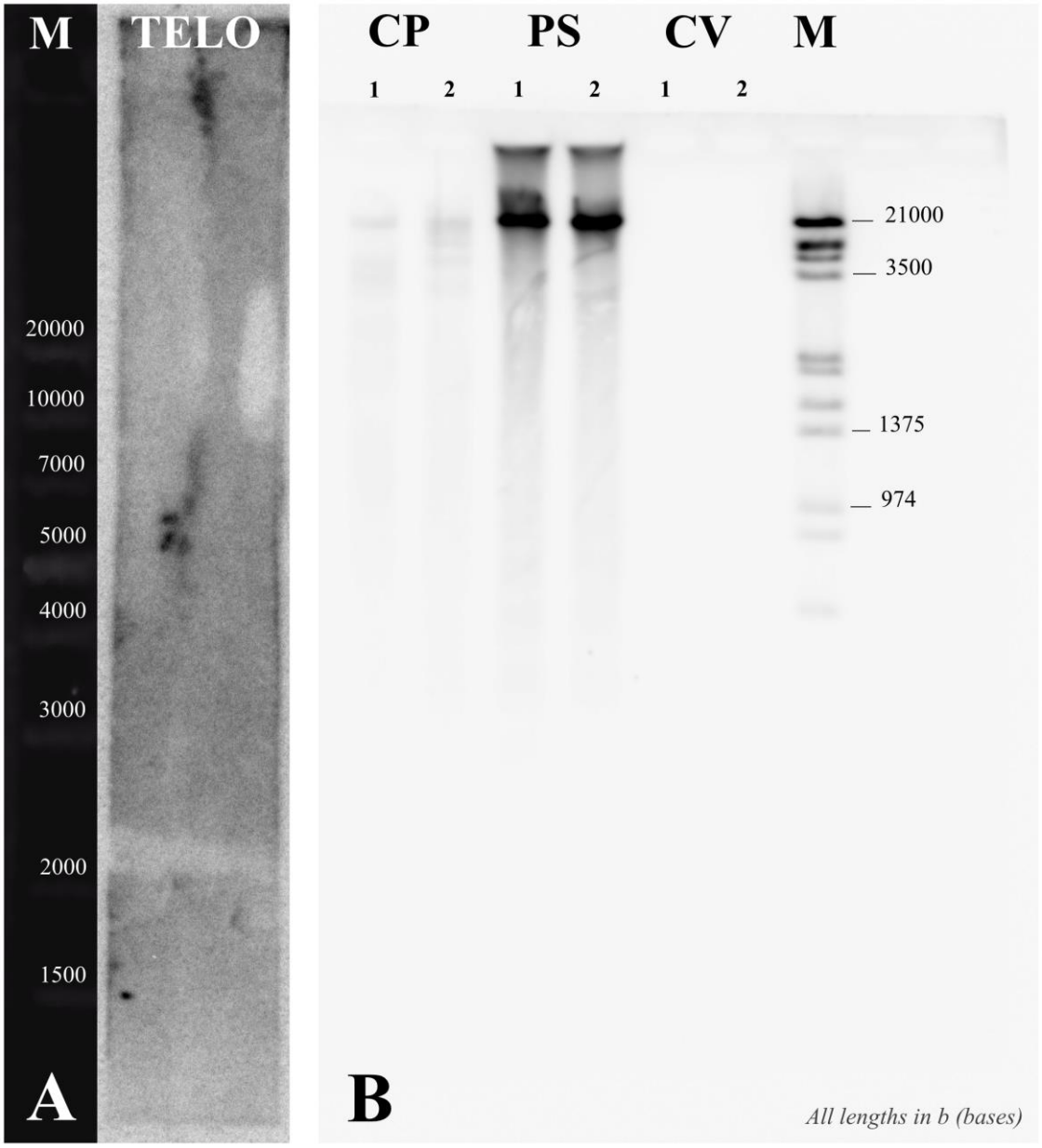

Fig.13: Southern blot using radioactive and DIG-labeled telomere probes. **A** – The membrane using Alpha-^{32}P-dATP-labeled telomere probe. No signal was detected. The exposition time was 36 h. **B** – The membrane using DIG-labeled telomere probe. No positive signal for *C. velia* (CV) was detected, while the intense signal was gained in case of *P. sativum* (PS) positive control and also very weak signal in case of *C. pomonella* (CP) negative control. The time of exposition was 2 min. DNA size labels are shown in both marker lanes (M).

5. Discussion

The determination of ploidy and the total number of chromosomes of an organism can be very contributive to the understanding of how the genome information is organized. The principal task of this thesis was to perform these chromosome analyses in *C. velia* by the use of fluorescence *in situ* hybridization.

The first issue I addressed was the perforation of the cell wall and the subsequent isolation of nuclei. Without these optimizations, the probes or anti-hapten-HRP-labeled conjugate failed to penetrate the thick cell wall of *C. velia* and hybridize with the target sequences of interest (see Fig.6). Pernthalern and Pernthalern (2007) faced the same difficulties in varied environmental microbes. In *C. velia*, this issue was exemplified in the nucleic acid staining. The isolated nuclei were significantly better DAPI-stained in comparison to the cell-located nuclei (see Fig.5, 1a). Cell wall permeabilization was essential for a proper DAPI stain of intracellular DNA material (Chazotte, 2010a). For the staining of the nuclei in non-lysed or even vital cells, Hoechst stain is more suitable (Chazotte, 2010b), but we experienced difficulties with Hoechst staining in *C. velia* as well (Cihlář, personal communication). There are many cells from which the nuclei have already been isolated – ranging from plant to human cells (Bhargava, 1971; Poglitsch et al., 2011), but a specific enzymatic mix was needed to remove *C. velia* cell wall. I tried previously described mixtures (Chen et al., 2008; Pan et al., 2013) but none was working with *C. velia*. Eventually, based on cell wall chemical composition, I chose to use the LLYC enzyme combination and was able to digest about fifty percent of the treated cell culture. I did not intend to purify the nuclei isolate from the cell residues, as this impure culture was sufficient for the preparation of slides and subsequent FISH performance.

The improvement of the nuclei isolation method is planned, nevertheless. The obtaining of the pure nuclei isolate should be possible with the use of Percoll or sucrose discontinual gradients followed by ultracentrifugation (Folta and Kaufman, 2006; Sikorskaite et al., 2013). If a protoplast isolation protocol is at hand, the following steps for the separation of nuclei from other cellular structures should be tested. Ohyama et al. (1977) avoided mechanical protoplast disruption, and used 0.5% Triton X-100 together with low speed centrifugation to obtain pure nuclear suspension. In comparison, Tallman and Reeck (1980) let protoplasts burst in hypotonic buffer. The isolated organelles were then purified from protoplast residues by sample filtration through 8 μm pore-size membrane filter. Then, the

nuclei fraction was separated by centrifugation in a discontinuous sucrose gradient. Saxena et al (1985) disrupted protoplasts, suspended in nuclei isolation buffer (10mM 2-(N-morpholino) ethanesulfonic acid, 0.2% sucrose and 0.01% Triton X-100, pH 5.3 and temperature 4 °C), by a table grinder or gauge needle. The protoplast residues were filtered out by Miracloth filter and nuclei were subsequently purified by use of polycarbonate filters.

Alternatively, an improved system of hybridization using nanobodies could be considered (Kaplan and Ewers, 2015). Nanobodies are small binders (about 15 kDa) that can readily penetrate the cell wall without previous digestion. In this protocol, all cell structures are preserved and valuable time is saved. Nonetheless, the nanobodies come from the heavy chain of camelid immunoglobulins, so they are solely protein-antigen specific, what makes them suitable for the use in diagnostics and biomedical applications (Hassanzadeh-Ghassabeh et al., 2013; Gu et al., 2014; Fridy et al., 2014), but inapplicable in our research.

For the *C. velia* ploidy examination, I decided to utilize the tyramide signal amplification – fluorescence *in situ* hybridization, allowing up to one-thousand-times signal amplification (Schriml et al., 1999). For instance, Paladino et al. (2014) utilized TSA-FISH to map the single copy genes in the codling moth, *Cydia pomonella*. In our case, the signals resulting from standard FISH were expected to be too weak to appear, since the lengths of the probes for single copy genes were about 500 bp, and there was only one target sequence of interest per one chromosome. Increasing probe lengths would possibly interfere with their penetrance across the cellular or nuclear membrane. On the other hand, shorter probes show lower specificity of hybridization and the intensity of the resulting signal could be greatly reduced. The use of TSA-FISH and 500b-long probes proved to be the optimal option and made possible to assay the ploidy of *C. velia*. I observed one signal in all nuclei with apparently hybridized SC probes (Fig.7 and Fig.8). I show that *C. velia* is indeed a haploid organism, at least in the stages I managed to observe.

Before employing FISH, our team attempted to examine ploidy in *C. velia* by means of flow cytometry. The method, which played fundamental role in chromosome sorting during the human genome sequencing (Lander et al., 2001) and has numerous applications nowadays, relies on reading fluorescence of single stained or unstained cells in a laser-illuminated chamber. However, flow cytometry proved as the very deceitful method for *C. velia*, when the total genome size was initially estimated to 10 Mb (Oborník et al., 2009). This was soon disproven by next-generation sequencing, determining the *C. velia* genome size to 193 Mb

(Woo et al., 2015). This huge difference could be caused by an inappropriate reference standard, which is crucial for the exact flow cytometry measurement (Tiersch et al., 1989), or because of the inability of the dye to properly stain the genetic material, similar to what we experienced during whole-cell staining. In addition, flow cytometry failed to determine ploidy of cultures with induced zoosporogenesis. SYBR Green stained the entire cell material, probably causing misrepresentation of the results (Cihlář, personal communication). The cell wall digestion procedure developed during this might have enabled a better staining, but applicability to zooflagellates is questionable. Either way, in comparison to flow cytometry, FISH proved more accurate in ploidy examination (Kipp et al, 2011).

I show that *C. velia* has a haploid stage, similarly to Apicomplexa and Dinoflagellata from the subgroup Myzozoa and the majority of other representatives of group Alveolata (Taylor, 1987; Margulis, 1990). On the other hand, *C. velia*, unlike Apicomplexa and Dinoflagellata, quite possibly lacks an interim diploid stadium in its life cycle, or, the diploid stadium could emerge only for a very short time or just sporadically (Taylor, 1987; Margulis, 1990; Oborník et al., 2011; Oborník et al., 2012). In diploid organisms, the sexual processes allow alleles to be mixed, giving organism the additional capacity to react to the environmental influences with novel combinations of traits (Alberts et al., 2002). Apicomplexa and parasitic dinoflagellates such as *Oxyrrhis marina* (Lowel et al., 2010) and *Perkinsus marinus* (Mackin et al., 1950) have co-evolved in an incessant arms race with their hosts' immune systems. In this unwelcoming interaction, the parasites most likely had to ceaselessly adjust their surface receptors to maintain entry to host cells and to escape host immunity if necessary (Cowman and Crabb, 2006; Frank, 2002). Without sexuality, they would have lost additional means of exchanging variation. Should we consider *C. velia* a coral symbiont (Janouškovec et al., 2012a,b) that has little problem to penetrate the cells of its partner (Cumbo et al., 2013) and thrive there, it is possible that *C. velia* lost the interim diploid stadium as unnecessary and relies on vegetative life cycle.

In a first attempt to study the *C. velia* chromosome number, I used telomere probe against *C. velia* telomere sequence TTTAGGG (Fulnečková et al., 2013) and direct FISH. Although this method is usually sufficient for the examination of the repetitive telomere sequences (Hacia et al., 1999), it was unsuccessful in case of *C. velia*. In the end, the enhanced performance of TSA-FISH enabled the examination of telomeres in *C. velia*. Consistently, there were two fluorescent signals per nucleus (Fig.9 and Fig.10) indicating that *C. velia*

possesses only one chromosome in total. It would be quite a large chromosome with the total size of 193 Mb, however, certain representatives of dinoflagellates undoubtedly possess even larger chromosomes, since the size of their genomes ranges from 1,500 to 245,000 Mb (Hackett et al., 2004; Wisecaver and Hackett, 2011), while containing no more than 200 chromosomes (Hackett et al., 2004). If that is the case, *C. velia* would be the first asexual eukaryote with a single chromosome. To date, the only other example in sexual organisms is the haploid male of ant *Myrmecia pilosula* (Crosland and Crozier, 1986). The second possible interpretation of this two-signal result could be chromosome clustering. During interphase, the telomeres of some species are tightly bound by proteins into so-called clusters linked to the nuclear envelope via protein interactions (Kanoh, 2013). After the FISH hybridization, these clusters could consequently appear as a single dot per each cluster, i.e. the chromosome end.

I tried to test these two hypotheses by using Southern hybridization. Following restriction cleavage in subtelomeric region, we would expect one band or two tightly overlapping bands for the single chromosome and more bands or a smear if telomere clusters are formed from a higher number of chromosomes. Probe sensitivity, hybridization specificity and gDNA quality were verified by dot blot, a materially undemanding and non-time-consuming technique (Mearns et al., 1988). For Southern blotting, I compared radioactive and DIG labeling of probe. Radioactivity is considered to be the most sensitive labeling technique (Osborn, 2010), but DIG labeling sometimes produces less background. In both cases however, I was not able to obtain positive signal for the *C. velia* telomere sequence. Notably, I obtained intense signal for the positive control – TTTAGGG telomeric sequence of *P. sativum* and even very weak signal for the negative control – TTAGG telomeric sequence of *C. pomonella*. The positive control indicates not only the functionality of the protocol, but also a non-mutated standard telomere sequence of *P. sativum*. The weak signal in the negative control is most likely caused by unspecific hybridization of telomere probe with the telomere sequence of *C. pomonella* and could be caused by insufficient stringency of the washing steps. The inability of telomere probe to hybridize with *C. velia* gDNA after Southern blot was surprising, since I observed a very strong hybridization signal from the dot blot. Nevertheless, DNA used in dot blot technique is simply denatured and then directly loaded onto the membrane (Pallás et al., 1998), without the need for nuclease digestion, separation and blotting, where samples can be lost or damaged. I used specific "zero cutter" restriction endonucleases, which cut within subtelomeric regions rather than the target

sequence of interest – the telomere. However, star cleavage in telomere sequence cannot be ruled out, making the target artificially shorter and highly fragmented so signal can no longer be measured – it would be below the detection limit. This is not an issue during dot blot and TSA-FISH techniques, where DNA is chemically undamaged. Additionally, partial depurination, when purine bases adenine and guanine are hydrolyzed at N-glycosyl bonds to deoxyribose (Alberts et al., 2002), is used to alleviate the migration of large DNA fragments. The level of depurination is directly proportional to pH and the time of incubation, and single-stranded DNA such as telomeres may be more seriously affected than dsDNA (An et al., 2014). It is therefore possible that telomeres could be radically damaged, or even lost just before blotting. It could be also a problem of washing stringency, but we consider this as implausible as the negative control still produced background signal.

Another possible explanation could be unconventionally short or unorthodox *C. velia* telomere sequences. Low numbers of repetitive telomere monomers, or appearance of inserted sequences between short arrays of monomers (Lin et al., 2008) would explain our failure to employ direct FISH. Potentially, the telomere sequence of *C. velia* could be mutated in particular percentage, resulting in weakened hybridization (Tayeh et al., 2009) with a different limit of detection for Southern blot and FISH. The level of detection is greatly influenced by differences between the washing steps and input form of DNA in Southern blot and FISH. A single base mutation in short probe sequence can significantly decrease the melting temperature. A single mutation in the seven-bases-long telomere monomer could lead to extreme decrease in the melting temperature and washing away of probe. Single base mutations have already been discovered in *C. velia* telomeres (Fulnečková et al., 2013). Short and non-conserved telomeres could also hamper the detection of multiple small chromosomes that do not produce sufficient signal when electrophoretically separated.

Pulse field electrophoresis is an alternative separation method for undigested native DNA such as chromosomes, although the upper limit is about 10 Mb in size (Herschleb et al., 2007). Larger molecules might not be properly separated, resulting in data misrepresentation. Therefore, pulse-field electrophoresis would only be favorable in case *C. velia* genome consists of numerous smaller chromosomes.

To conclude, we made substantial progress in understanding *C. velia* genetic information. To gain further insight, improvement in the nuclei isolation method must be achieved to harvest

enriched nuclei suspension. Further analyses such as Southern blot and pulse field electrophoresis with modified conditions are needed to unambiguously determine the chromosome number.

6. Conclusion

- I managed to isolate intact interphase nuclei by a combination of enzymatic and mechanical treatment.

- I successfully utilized tyramide signal amplification – fluorescence *in situ* hybridization and Southern blot to examine *C. velia* ploidy. We show *C. velia* is a haploid organism.

- I employed TSA-FISH to determine the total chromosome number in *C. velia*. However, the results are ambiguous, indicating that a single large chromosome is present, or the telomeres are organized into clusters in interphase nuclei.

- We suggest optimization and performance of techniques to clarify the ambiguities, namely Southern blotting, pulse-field electrophoresis and optimization of nuclei isolation.

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