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Genome size stability of dried tissues of selected genera of ferns and lycophytes

Bachelor thesis

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Annotation

Flow cytometry is an important method in various science fields, whereas in botany it is mostly used for genome size and ploidy estimation. In the last two decades research was conducted on the influence of different storage conditions on the quality of flow cytometry histograms and reliability of the genome size estimation for angiosperms. Thus, this thesis is focused on the influence of storage time and different types of storage by desiccation on the quality of the obtained flow cytometry histograms and genome size estimation reliability in selected ferns and lycophytes.

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, …… …..…….

Ondřej Šimeček

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Table of contents

1 Introduction

Plant nuclear genome size (the total DNA amount in a cell nucleus) may be highly important for understanding their biodiversity, hybridisation, ecology, and breeding (Corneillie et al., 2019; Suda et al., 2015). With its huge variations, genome size estimation is one of the most important characteristics in botany. It can be carried out by methods based on intensity measurement of selective DNA binding dye, whereas Feulgen image analysis densitometry (FIA) and flow cytometry (FCM) are of the greatest importance in botany (Corneillie et al., 2019; Doležel et al., 1998, 2007; Maule, 1998; Suda & Trávníček, 2006). However, FCM is rather more frequently used, for its simplicity, high throughput analysis and reliability (Greilhuber, 2008).

One of the outputs of FCM analyses is the fluorescence intensity, which is directly proportional to the genome size with the result typically visualized in a histogram. As in these analyses only the relative intensity is measured, an internal standard with a well-defined genome size must also be employed to estimate the genome size of the sample (Temsch et al., 2021). The material used as an internal standard is most frequently nuclei suspension from a living plant since the analyses of fresh nuclei usually provide the most reliable information. Most ideally the nuclei of the sample should be from a fresh plant as well, though due to various reasons sometimes the samples are required to be conserved for a longer time (Čertner et al., 2022).

The conservation of the plant material may be performed in several ways, e.g., conservation of the nuclei suspension by deep freezing, placing the plant sample into herbarium or drying it in a silica gel (Čertner et al., 2022). Unfortunately, the FCM analysis of those conserved samples are usually not so reliable and informative, as the nuclei and the DNA chemically degrade and, eventually, the fluorescent signal of the sample may completely vanish (Bainard et al., 2011a; Doležel & Bartoš, 2005; Suda & Trávníček, 2006). Additionally, literature comparison reveals that degradation is species-specific and therefore rather unpredictable.

Until now, the genome stability of dried samples was mostly investigated among flowering plants (angiosperms) (Šmarda & Stančík, 2006; Suda et al., 2007; Suda & Trávníček, 2006), although less research can be found among other groups (Bainard et al., 2011a). Thus, this work focuses on the genome stability FCM measurements of common species of ferns and lycophytes – spore bearing vascular plants (ferns, lycophytes) under different conservation

strategies. The work should elucidate how long and with what desiccation technique selected fern and lycophyte species can be preserved to provide precise results during FCM analysis. Additionally, it will show differences among selected species, which may be useful when selecting the best conservation techniques.

1.1 Flow cytometry

1.1.1 Principle

Flow cytometry is currently the most common method applicable for genome size estimation and particles evaluation. It allows high throughput analysis of single cells or particles in a short time frame (Doležel et al., 2007a; Loureiro, 2007; Rieseberg et al., 2001). The cells (particles), which are usually stained by a fluorochrome, are aligned in the flow cytometer into a single file by a liquid sheath fluid (Doležel et al., 2007a; Givan, 2011; Rieseberg et al., 2001). Subsequently, the cells (particles) are enlightened by an intensive light and in the following few microseconds the scattered light, emitted light, and other signals are detected and processed (Doležel et al., 2007a; Rieseberg et al., 2001). Thus the main advantages of the principle of flow cytometry are that it can analyse huge number of cells (particles) in a short period of time, the measurements are multiparametric, and finally, it uses every cell (particle) as a multiparametric measurement unit, making the results statistically strong (Doležel et al., 2007a; Rieseberg et al., 2001).

1.1.2 Instrumentation

Flow cytometer machines are complex devices whose components may be principally divided into three categories: fluidics, optics and electronics. Since the number of parameters measured is quite high, bioinformatics should be as well considered as a last and crucial component in flow cytometry (Doležel et al., 2007a; Givan, 2011).

1.1.2.1 Fluidics

The main purpose of **fluidics** is to transport the sample cells (particles) through the flow cytometer, align them, pass them through the light beam and carry them into the waste (Doležel et al., 2007a). An essential component of the fluidics is a flow chamber, where the sample suspension joins the sheath fluid (Doležel et al., 2007a; Givan, 2011). Here, the sample suspension flows through a sample injection tube, while the sheath fluid flows faster around the sample injection tube and creates a drag on the sample cells (particles) (Doležel et al., 2007a; Givan, 2011). This drag together with the decreasing diameter of the cross-section of the flow chamber aligns the previously disorganized cells (particles)

into an organized row within central core of the coaxial flow, where this effect is known as hydrodynamical focusing (Doležel et al., 2007a; Givan, 2011). However, this phenomenon works only when the sample suspension is close to sheath fluid in viscosity and hydrophobicity (Golden et al., 2012). Additionally, the hydrodynamical focusing requires a laminar flow of the sheath fluid, which can be described by Reynolds equation (Equation 1),

$$R_e = \frac{v\rho d}{\eta} \qquad \text{(Equation 1)}$$

where R_e is the Reynolds number [dimensionless], v is the velocity of the fluid [$m \cdot s^{-1}$], d is the diameter of the tube [m], ρ is the density of the fluid [$kg \cdot m^{-3}$], η is the viscosity of the fluid [$kg \cdot m^{-1} \cdot s^{-1}$] (Doležel et al., 2007a; Mansour et al., 2020). For R_e below 2100–2300 the flow is always laminar, while for R_e above 2300 the flow can already be turbulent, thus, the particles can be disorganized (Doležel et al., 2007a; Reynolds, 1883). Followingly, after the cells (particles) align, they are intercepted with a laser beam, the signals from them are measured and, finally, they flow with the sheath fluid to a waste container (Givan, 2011).

The driving force of sample injection and sheath fluid flow can vary in different flow cytometers. The first possibility is to force both the sample and sheath fluid to flow by applying air pressure over both liquids. The second possibility is to use automated motor-driven syringes to regulate the flow. Finally, the last option is to use a peristaltic pump (Doležel et al., 2007a; Givan, 2011). As mentioned above, the sheath fluid flows few orders of magnitude faster than the sample. Depending on the ratio between the velocity of the sheath fluid and the velocity of the sample solution, the diameter of the central core, containing the sample solution, is described by Equation 2,

$$d_{cc} = \sqrt{\frac{v_{sample}}{v_{sheath}}}\, d_n \qquad \text{(Equation 2)}$$

where d_{cc} is the diameter of the central core [m], v_{sample} is the sample flow velocity [$m \cdot s^{-1}$], v_{sheath} is the sheath fluid flow velocity [$m \cdot s^{-1}$] and d_n is the diameter of both sample (central core) and sheath fluid [m] (Doležel et al., 2007a).

1.1.2.2 Optic systems

Optic systems (optics) include the light source used in the flow cytometer together with lenses used to collect the signals from excited cells (particles) and filters used to select for the desired wavelength (Doležel et al., 2007a; Givan, 2011).

Older types of flow cytometers typically use a mercury arc lamp producing the whole spectrum of wavelengths, which need to be further filtered by optical filters prior to the sample illumination (Doležel et al., 2007a; Picot et al., 2012). Nowadays, lasers are used in majority of modern flow cytometers, because unlike mercury arc lamps they are monochromatic light sources without the necessity of further filtration. (Doležel et al., 2007a; Telford, 2011). Additionally, they are more efficient, they provide coherent light, and the shape of the light beam can be well controlled (Telford, 2011). As the laser light source properties dictate the overall efficiency and sensitivity of the flow cytometer instrument, they should be carefully controlled (Doležel et al., 2007a). One of such key properties is so called laser noise, which is the measure of fluctuations of the properties of the laser beam between individual illumination of the cells (particles) in flow cytometer. For less precise measurements with higher variability, laser with quite high noise can be used, though for more precise measurements, such as the DNA content measurement, low-noise lasers are needed (Telford, 2011). Additionally, for optimal measurements the laser should operate in transverse emission mode 00 (TEM 00), the shape of the light beam should be elliptical (ca 15×60 μm) and the laser should have high laser power and pointing stability (Doležel et al., 2007a; Telford, 2011).

1.1.2.3 Electronics

After the cells (particles) are excited with a monochromatic light and they emit, scatter, or refract light back, **electronic systems (electronics)** start to play an important role. The first component of the electronics are photodetectors, which convert the signal from photon into electrons (current), which can be processed by further components (Doležel et al., 2007a; Givan, 2011). There are generally two types of photodetectors used in flow cytometry, photodiodes, and photomultiplier tubes (PMTs). Photodiodes are usually made of semiconductors typically based on silicon. Current is created in photodiodes due to photoelectric effect, so the strength of the current signal is directly proportional to the strength of the incoming signal (photons). Thus, it is obvious that photodiodes may not be suitable for detecting light of lower intensities (Doležel et al., 2007a; Givan, 2011; Popovská, 2009). However, the problem of the signal strength is solved by the PMTs, where the light first enters the detector through a glass or quartz window. Subsequently, the light interacts with a cathode and an electron is emitted per each photon due to photoelectric effect (Doležel et al., 2007a; Foord et al., 1969). The emitted photons are then accelerated by an electric potential towards a first dynode with higher positive charge,

which leads to emission of higher numbers of electrons. Those electrons are accelerated to a second dynode with even higher positive charge, and this cycle repeats until the electrons reach the last dynode and they are collected. So, even with a low initial intensity of the light, strong signals (up to 10^6 times stronger) may be collected (Doležel et al., 2007a; Foord et al., 1969). Nevertheless, for each wavelength a different photodetector is required, so four to five photodetectors have typically been implemented in the cytometers, although recently flow cytometers started to implement up to 10 photodetectors, usually in sterically favourable arrangements (e.g. circle) (Doležel et al., 2007a; Givan, 2011). The light measured by the photodetectors may have several origins. First, we can measure the forward scattered light (FSC), which is light scattered or refracted in the direction of the laser beam by small angles. As this light has quite high intensity, it is usually measured by a photodiode (Doležel et al., 2007a; Givan, 2011). The light scattered by big angles from the laser beam is collected by lenses and detected by PMTs in 90° angle and is called the side scattered light (SSC). Finally, the light may also originate from the fluorescence of the fluorochrome, where this light can have quite low intensity, so it is typically detected by PMTs (Doležel et al., 2007a; Givan, 2011).

Another crucial component in a cytometer is an analogue-to-digital converter (ADC), which converts the analogue continuous voltage signal into a discrete digital signal (Doležel et al., 2007a; Givan, 2011). The ADC, though, can only convert the data at certain time ranges, otherwise it would be overloaded with data. Therefore a discriminatory circuit is always included in the flow cytometer, which activates the data collection apparatus (including ADC) when a threshold signal voltage is exceeded (Doležel et al., 2007a; Givan, 2011). Additionally, preceding the digitalisation of the signal, it is amplified either in linear or logarithmic manner (Givan, 2011). Linear amplifiers create outputs directly proportional to their inputs, though if the signals are different by orders of magnitude, logarithmic amplification is more appropriate (Doležel et al., 2007a).

1.1.2.4 Bioinformatics

After the signal is converted from the analogue to the digital domain, it is passed to a computer, where it is stored as a Flow Cytometry Standard file (FCS) (Doležel et al., 2007a; Givan, 2011; Murphy & Chused, 1984). The original FCS file was encoded in American Standard Code for Information Interchange (ASCII), though, the current versions are coded in Unicode instead (Doležel et al., 2007a; Murphy & Chused, 1984; Seamer et al., 1997). The file itself contains 3 compulsory segments and 1 optional segment.

The first segment is a unique identifier (header), which identifies the type of the file (FCS) and the version of FCS used. Additionally, by numerical values it points out the position of the second segment. The second segment is called the text segment, and it is composed of keywords, which characterize the third (data) segment. The latter segment presents numerical values, specified by the text segment. Finally, the last (analysis) segment is optional and can contain the information about the analysis in a text format (Doležel et al., 2007a; Murphy & Chused, 1984).

For graphical representation of the results, histograms and dot plots are commonly used. (Doležel et al., 2007a; Givan, 2011; Rieseberg et al., 2001). Histograms plot the cell count against one or more of the chosen parameters, while dot plots (or possibly density or contour plots) are used for correlation of any two or more parameters (Doležel et al., 2007a; Doležel & Bartoš, 2005; Givan, 2011)). Typically, one may also interpret the result of the FCM analysis from those plots (Doležel & Bartoš, 2005; Dressier & Seamert, 1994). For histogram recording either a linear or logarithmic x-axis (fluorescence intensity) may be used. While the advantage of linear x-axis is that the distances between peaks directly represent genome size differences, and that CV values are recorded, logarithmic peaks are useful for measurements of values which are far apart each other. Those types of recording can be converted back and forth only partially (i.e. not all the data are recovered) (Koutecký et al., 2023).

1.1.3 Genome size estimation by flow cytometry

Besides other important uses of FCM, it can be used for genome size estimation, as mentioned above (Doležel & Bartoš, 2005; Doležel & Greilhuber, 2010; Suda & Trávníček, 2006). For this use isolated nuclei are required in the analysed solution, while the DNA fluorescence of these nuclei is measured. As DNA excitation and emission happens in UV spectrum, to obtain fluorescence in visible spectrum, DNA binding fluorophores must be added prior to the analysis. Finally, as the fluorescence is measured relatively, a reference standard with well-defined genome size is also added to the analysed solution prior to the extraction of the nuclei (Doležel & Bartoš, 2005; Doležel & Greilhuber, 2010; Vaya et al., 2010).

1.1.3.1 Nuclei isolation buffers

The use of the right buffer is essential for sample preparation and correctness of the obtained results. There are various compositions of the buffers, though, the crucial components remain the same (Loureiro et al., 2006; Sadhu et al., 2016). Those components include organic

pH buffers to hold the pH at certain level (Tris, MOPS, HEPES etc.), chromatin stabilizers (magnesium salts, spermin, spremidin etc.), chelating agents to inhibit nucleases through Mg^{2+} and Ca^{2+} binding (EDTA, citrate etc.), inorganic salts to achieve desired ionic strength (KCl, NaCl etc.) and detergents to release the nuclei from the cells (Tween 20, Triton X-100 etc.) (Loureiro et al., 2006, 2021; Sadhu et al., 2016). Additionally, in some buffers an organic reductant and polyvinylpyrrolidones are included to bind polyphenolic compounds (Loureiro et al., 2021). Among the buffers the Otto's buffer is an exception (Otto, 1990). As opposed to the common buffers composed of two solutions, Otto I contains the detergent (Tween 20) and the chelating agent (sodium citrate) and Otto II contains inorganic phosphate buffer, reductant (2-thioethanol) and a fluorophore (Hooghvorst et al., 2019; Loureiro et al., 2021; Suda & Trávníček, 2006).

1.1.3.2 Fluorochromes

To obtain fluorescence from the DNA, fluorochrome staining must be applied on the nuclei. To obtain precise results, those fluorochromes must bind specifically to DNA and in constant ratio (Givan, 2011; Loureiro et al., 2021). Generally, we may divide the fluorescent stains into two classes: (1) intercalary fluorochromes used to estimate total genome content, and (2) minor groove binding base-specific fluorophores used to estimate the AT/GC ratio (Doležel et al., 2007a; Doležel & Greilhuber, 2010; Loureiro et al., 2021). The stains from the first group are planar molecules, resembling the whole DNA base pair (e.g. ethidium bromide (EB) or propidium iodide (PI) etc.). As those dyes bind to the DNA independently on the AT/GC ratio, they are suitable for total genome size estimation in picograms or mega base pairs units (Doležel et al., 2007a; Loureiro et al., 2021). Unfortunately, heterochromatin structures with significant steric hindrance may not be stained with the intercalating dyes, leading to possibly precise, but inaccurate results (Loureiro et al., 2021).

Opposingly, the second type of dyes bind to the minor grove of DNA double helix with specificity to AT-rich (e.g. DAPI, Hoechst dyes) or GC-rich (e. g. olivomycin, mithramycin) binding sites (Doležel et al., 2007a; Loureiro et al., 2021; Tanious et al., 1992). Both groups of dyes have the biggest affinity towards tetramers of their corresponding binding sites (Breusegem et al., 2002; Carpenter et al., 1993). Additionally, in the first group of dyes the binding to GC sites is prevented by the guanin amino group, while in the second group the binding specificity is ensured by higher affinity constants (Carpenter et al., 1993; Tanious et al., 1992). Finally, the GC specific dyes require a divalent metal ion as well to emit fluorescence of sufficient intensity (Beniaminov et al., 2020; Loureiro et al., 2021).

1.1.3.3 Standardization

As already mentioned above, the measured fluorescence of the fluorophore is only relative, so there is a need for a standard, and there are several types of them: (1) abiotic (such as fluorescent beads) (2) external biological standard, which is measured separately from the sample under similar conditions (3) internal biological standard, which is measured under the exact same conditions as the sample (Doležel et al., 2007a; Tiersch et al., 1989). It is obvious that the internal biological standard will give the most precise results, due to having the same conditions of the measurement as the sample. Ideally, the internal biological standard should have precisely measured genome size different from the sample, but close enough to behave similarly under the same experimental conditions (Doležel et al., 2007a; Doležel & Bartoš, 2005). Additionally, the standard should also be genetically stable and available in sufficient amount. As the possible samples can vary greatly in genome size, one typically needs a whole set of standards (Doležel et al., 1998). Finally, an advantage of some internal biological standards is that 4C (double DNA content than diploid) peaks are measured in addition to the 2C (diploid DNA content) peaks, as significant number of cells is in the G_2 phase of the cell cycle (Čertner et al., 2022).

1.1.4 Other uses of flow cytometry

So far, mainly the use of genome size estimation by FCM was considered above, however FCM has much more practical applications. One of those is cell sorting, which is frequently used in various fields of science (Doležel et al., 2007a; Osborne, 2011). Here, the cells are measured in FCM device, subsequently, the mother solution is broken down into small droplets containing single cells and those droplets are charged (or not charged) depending on the measured parameters. Finally, the cells are deflected into different vials by applied electric field (Davey & Kell, 1996; Doležel et al., 2007a; Osborne, 2011; Picot et al., 2012). The concept of sorting may be further extended for other particles than cells, for instance, nuclei or chromosomes, as this may simplify genome sequencing and chromosome proteomic analysis (Doležel et al., 2021).

In biotechnology, flow cytometry may be helpful in protein engineering, as the cells producing the desired protein may be marked by specific fluorescent probes (Wójcik et al., 2015). With this, cells, which either produce the desired protein or do not produce the desired protein, may be counted and (or) sorted, as this may help in screening for different protein variants, screening and counting cells which underwent successful transformation, or just simply in fast sorting and protein production for research or medicinal uses (Wójcik et al., 2015).

Another possible application of FCM is measuring cell viability, used in microbiology and food industry (Wilkinson, 2018). For this purpose, cells are stained by differential fluorescent stain distinguishing living and dead cells, which allows the flow cytometer itself to count and (or) sort the cells (Comas-Riu & Rius, 2009; Wilkinson, 2018). The advantage of this method is its speed and overcoming of the inconveniences of plate-culturing methods (Comas-Riu & Rius, 2009).

Finally, flow cytometrical methods are quite frequently used in medicine. As mentioned above, it can be used for DNA or RNA analysis to diagnose genetical disorders and to prognose cancer (Wijnaendts et al., 1993). In blood and bone marrow analysis, the flow cytometry is used for counting and sorting white blood cells for the purpose of the proper diagnoses of diseases (Della Porta et al., 2011; Van De Geijn et al., 2011). Finally, in some cases flow cytometry may be also useful to analyse cerebrospinal fluid, e. g., in lymphoblastic leukaemia or bacterial meningitis (Davis & Westerman, 2014; Saito et al., 2005).

1.2 FCM genome size estimation in plants

As we could see in chapter 1.1.4, flow cytometry is a method with wide uses in many fields. However, while still using the same method with slight adjustment in the instrumental set-up, the scientific field specifics and the experimental specifics need to be considered to obtain correct and precise results. Thus, in this chapter the experimental considerations for genome size estimation in plants are discussed.

1.2.1 Plant internal standards

The internal standards recommended for plants should ideally contain 2C (G_1) and 4C (G_2) peaks, as this greatly simplifies the subsequent peak identification. The C value of the standard should be close to that of target nuclei, though not overlapping the target nuclei (Johnston et al., 1999; Temsch et al., 2021). This parameter is very important, as the 2C genome size within plants can vary ranging from 0.061 Gbp (appr. 0.062 pg) in *Genlisea tuberosa* to appr. 297.76 Gbp (304.46 pg) in *Paris japonica* (Doležel et al., 2003; Fleischmann et al., 2014; Pellicer et al., 2010). Finally, the chromatin structure of both should be comparable, as it minimizes interferences (Johnston et al., 1999; Temsch et al., 2021). The 2C values of the standards can also be estimated according to Equation 3 in FCM measurement with standards with well determined genome sizes ("golden standards"), such as the human leucocytes with 7 pg 2C value (Tiersch et al., 1989).

$$2C_{standard} = 2C_{human} \cdot \left(\frac{F_{G1/G0,\ standard}}{F_{G1/G0,\ human}}\right) \quad \text{(Equation 3)}$$

Here, $2C_{standard}$ and $2C_{human}$ are the 2C values of the standard and human respectively and $F_{G1/G0,\ standard}$ and $F_{G1/G0,\ human}$ are the relative fluorescence intensities of cells in G1 or G0 phase of the standard and human respectively (Temsch et al., 2021).

The basis of the alternative and second most widespread cascade of internal standard values is the sequenced whole genome of *Oryza sativa* subsp. *japonica*, whose 2C value is 0.8 pg. Some of the most frequently used internal standards for both DAPI and PI FCM are listed below in Table 1 together with their C values in pg and genome sizes in Gbp.

Table 1: Standards commonly used for plant genome size estimation analysis in DAPI and PI flow cytometry with their 2C values and genome size based on human cells (2C = 7 pg), values with asterisk are those from literature, while the complementary value was recalculated according to the formula C value [pg] = genome size [Gbp]/0.978 (Doležel et al., 2003).

Standard	**2C value [pg]**	**Genome size [Gbp]**	**Reference**
Allium cepa 'Alice'	34.89*	34.12	(Temsch et al., 2021)
Bellis perennis	3.38*	3.31	(Schönswetter et al., 2007)
Chlorophytum comosum	24.14*	23.61	(Hornych et al., 2019)
Pisum sativum 'Ctirad'	9.09*	8.89	(Doležel et al., 1998)
Raphanus sativus 'Saxa'	1.11*	1.09	(Doležel et al., 2007a)
Solanum lycopersicum 'Stupnické polní rané'	1.96*	1.92	(Doležel et al., 2007a)
Secale cereale 'Daňkovské'	16.19*	15.83	(Doležel et al., 2007a)
Vicia faba 'Inovec'	26.90*	26.31	(Doležel et al., 1992)
Oryza sativa	0.795	0.778*	(Temsch et al., 2021)
Zea mays 'CE-777'	5.43*	5.31	(Lysak & Doležel, 1998)

1.2.2 Plant tissue selection and quantity

As mentioned in chapter 1.1.3, vital nuclei with DNA and feasible fluorophore are required for the genome size estimation, so theoretically any tissue containing those nuclei should be appropriate for the analysis (Doležel et al., 2007a). Nevertheless, due to secondary metabolites interfering with DNA staining, higher possibility of pathogen presence or tissue

degradation, nearly fully expanded leaves are preferable over other tissues. Developing tissues are not preferred as well as they are mitotically active (see chapter 1.2.3) and the DNA ploidy levels may greatly vary (Čertner et al., 2022; Doležel et al., 2007a). Finally, every plant species has its own specifics, so even though nearly developed leaves are a good solution for most of the experimental cases, some experiments need to use different tissues to get the best possible results, such as roots, stems, fruit skins, petals or even spores, endosperm and dry seeds (Čertner et al., 2022).

As for the tissue selection, the appropriate tissue quantity needs to be determined empirically, because there are quite huge variations in cell size (volume density of nuclei) among different plant species. Generally, for a typical measurement of the fresh leaf tissue described above, about 60 mg should be sufficient, yielding approximately 7500 nuclei (Čertner et al., 2022). Additionally, as the cell size frequently correlates positively with the genome size, larger tissue quantities should be measured for plants with huge genomes (e.g., ferns, some gymnosperms or monocots) (Beaulieu et al., 2008; Knight & Beaulieu, 2008).

1.2.3 Mitosis and endopolyploidy

To estimate genome size, it is necessary to assign peaks to sample and internal standard. While this may be a simple task for a tissue with low mitotic activity, in actively dividing tissues significant amount of G_2 phase nuclei may be present, making peak assignment more difficult (Čertner et al., 2022). However, using a standard consistently giving the G_2 phase peak may simplify the orientation of peak assignment (Doležel et al., 2007a).

Another phenomenon capable of altering fluorescence intensity of the peaks and adding peaks is endopolyploidy. In this phenomenon, the genome once or repeatedly duplicates without a creation of the new cell (i.e., endoreduplication), while creating nuclei with eight times more amount of DNA than the original cell (D'Amato, 1964) Unfortunately, the separation of the signal into multiple peaks leads to a decrease of the intensity in the diploid (2C) peak and as described above, it worsens the peak assignability (Čertner et al., 2022).

1.2.4 Interference with secondary metabolites

Secondary metabolites are non-essential compounds produced in the plant body, which may interfere with DNA staining procedures and alter the fluorescence intensity (Doležel et al., 2007a; Noirot, 2000; Price, 2000). The type and amount of those substances differs for different plant groups, but generally their higher concentration may be expected in some ferns, gymnosperms and also small percentage of angiosperms as Ericaceae,

Fagaceae, Geraniaceae or Rosaceae (Čertner et al., 2022). Under the presence of such compounds, fluorescence intensity may either increase, when the secondary metabolite binds to nuclei and attracts free fluorescing material (coatings of debris), or decrease, when the secondary metabolite binds to DNA dye-binding site and blocks the reaction with the fluorescent dye (fluorescent inhibitors) (Doležel et al., 2007a; Loureiro et al., 2021). The chemical structure and properties of the latter group is yet mostly unknown, though, phenolic compounds with active hydroxyl group are most prone to act as fluorescent inhibitors (Doležel et al., 2007a; Loureiro et al., 2021). In reduced state the hydroxyl groups form hydrogen bonds with the DNA (or proteins), so the fluorescent dye can still bind, as this binding is reversible and reaction equilibrium is established. However, in oxidized state phenolic compounds form quinones, which bind to DNA (or proteins) covalently leading to irreversible blockage of the dye binding site (Doležel et al., 2007a; Walle et al., 2003). Thus, to maintain the reversible state, reducing agents, such as β-mercaptoethanol, sodium metabisulfite or ascorbic acid, are added to the buffer solution. Additionally, for keeping the fluorescent inhibitors unbound to DNA, competing compounds, such as polyvinylpyrrolidones (PVPs), can be added. Those compounds then form hydrogen bonds with the fluorescent inhibitors leading to free DNA dye-binding sites (Čertner et al., 2022; Doležel et al., 2007a; Loureiro et al., 2021).

1.2.5 Applications for ferns and lycophytes

Pteridophyta (pteridophytes) are a polyphyletic taxonomical group referring to spore-bearing vascular plants, i.e., Lycopodiophyta (lycophytes) and Monilophyta (ferns) (Schneider & Schuettpelz, 2016). Even though ferns and lycophytes are studied less vigorously than angiosperms, there are multiple examples of the use of FCM genome size estimation for them in botany.

For instance, Clark et al. (2016) were able to analyse the genome sizes of 110 fern species with flow cytometry, where they proved that genome size and chromosome number is correlated in most fern taxa and reconstructed high chromosome numbers in all fern ancestors. In turn, Liu et al. (2019) analysed the genome sizes of the genus *Asplenium* by both collecting data from previous publications and by measuring by flow cytometry. Within this study the DNA content per chromosome or the predictability of genome size in polyploid *Asplenium* ferns was accessed. Finally, Fujiwara et al. (2023) showed that genome size evolution correlates with the diversification of various fern linages.

However, the perhaps most frequent use of genome estimation for ferns would be in the studies of polyploidy *(Asplenium)*, cytogeography (*Pteridium, Cystopteris)*, hybridization *(Dryopteris carthusiana* agg*., Polypodium)* (Bureš et al., 2003; Ekrt et al., 2022; Hornych et al., 2019; Schneider et al., 2017) Additionally, combination of polyploidization and hybridization could lead to reticulate evolution, observed also thanks to FCM in *Pteris fauriei* (Chao et al., 2022). Alternatively, FCM can be used for analysis of reproduction modes as apomixis (Ekrt & Koutecký, 2016; Ptáček et al., 2023).

For lycophytes the flow cytometry is rather less frequently used. Nevertheless, FCM measurements were primarly used for mostly the estimations of C values (Bainard et al., 2011b; Hanson & Leitch, 2002) but also for the study of hybridization and ploidy (Bennert et al., 2011).

Finally, FCM analysis are also often for the analysis of large sets of samples, such as in work of Fujiwara et al. (2023), which studied the correlation of diversification of fern linages with the genome size evolution.

1.3 Preservation of plant material

For DNA estimation with FCM analysis, best results are typically obtained with fresh plant materials. For this, several parts of a plant may be used, such as leaves, stems, roots, fruit skin or petals, though fresh leaves are recommended (Čertner et al., 2022). However, for studies requiring analysis of numerous samples this present an obstacle, as change in turgor may already alter the results of the FCM analysis (Suda & Trávníček, 2006). Several studies were conducted about this topic, such as the one of Bainard et al. (2011a), showing possible variation in genome estimates, when using PI staining in FCM. In turn some studies showed, that DNA may be estimated reliably, while using DAPI staining (Suda & Trávníček, 2006). Thus, this chapter introduces possible plant preservation techniques and assesses the compatibility with FCM DNA estimation.

1.3.1 Air desiccation

Preservation of plant species by free air drying or in herbarium, i.e., air drying under applied pressure at room temperature or slightly elevated temperature, are one of the oldest and most frequently used preservation techniques among different branches of botany (Besnard et al., 2014; Suda & Trávníček, 2006). For shorter periods of time (up to 20 months) angiosperm herbarium preserved samples in FCM measurements mostly result in peaks comparable with the fresh samples, though the reliability decreases, as the peak intensity

decreases and coefficient of variation (CV) decreases (Suda & Trávníček, 2006). For storage times around five years, only about half of the FCM measurements are reliable and for longer time periods (ranging up to 15 years) only a small fraction of the measured plant species can be successfully measured and for longer periods of time, generally, all the measurements are unsuccessful (Šmarda & Stančík, 2006; Viruel et al., 2019). However, Little et al. (2007) reported that after rehydration, reliable FCM histograms were obtained even for 15 years old herbarium samples.

1.3.2 Silica desiccation

Another approach of plant tissue preservation is its drying with silica gel beads as water sorbent, which can be done also under elevated temperature. As for the herbarium specimen, angiosperms provided decent peaks in shorter periods of time with slightly decreased intensity and increased CVs, which is suitable for reliable ploidy estimation with DAPI staining (Bainard, et al., 2011a; Suda & Trávníček, 2006). For about half a year old samples PI staining can be used as well, though for such storage times some of the measurement results may be already significantly shifted (Dyer et al., 2013; Sánchez-Jiménez et al., 2012). Finally, the time of reliable measurement may be prolonged by storage conditions, with deep freeze storage (storage in approximately -80 °C) prolonging the time the most (Suda & Trávníček, 2006).

1.3.3 Chemical preservation

Another method of tissue preservation is their chemical fixation in additive or non-additive fixatives, with the most commonly used being formaldehyde and ethanol/acetic acid solution respectively. Although, chemical fixation is quite compatible with ploidy estimation with DAPI staining, it is rather unsuitable for total genome size estimation with PI staining, as the fixatives may interfere with the stain and, thus, reduce the fluorescence intensity (Suda & Trávníček, 2006). Additionally, the chemical fixation is rather time consuming, as steps such as cell wall digestion or other enzymatic digestions are included (Hedley, 1989). Finally, it can be concluded that this method is rather useful in medicine, though while working with plants, chemical fixation is not a preferable method (Castro et al., 1993; Hedley, 1989; Overton & McCoy, 1994).

1.3.4 Nuclei protecting solutions

Similar to the chemical preservation is the use of protective solutions for nuclei. Typically, the nuclei are first isolated and then they are transferred to the protective solution,

where glycerol is probably the most frequently used protecting agent (Chiatante et al., 1990). Subsequently the suspension of the nuclei with the protecting agent are frozen, where a normal freezing (ca -20 °C) or deep-freezing (ca -80 °C) can be used (Chiatante et al., 1990; Vindeløv et al., 1983). Protective agents other than glycerol, such as citrate buffered dimethyl sulfoxide (DMSO) solution, are also possible, as shown by Vindeløv et al. (1983), though they only applied the solution for preserving whole cells, with subsequent deep-freezing.

These methods of protecting the nuclei can be used in the range of weeks to months (Chiatante et al., 1990; Kobrlová et al., 2020). The main disadvantage of this type of preservation is standardization, if there is no prior knowledge of genome size of the sample. Additionally, transportation may become quite challenging due to the necessity of keeping the sample frozen (Čertner et al., 2022).

1.3.5 Dry seeds and spores

Besides the above mentioned preservation methods, dormant dry seeds and spores may also be used in FCM genome size estimation after prolonged periods of time (Kuo et al., 2021). In the former, different anatomy of the seed in different plant groups (angiosperms and gymnosperms) has to be considered, whereas if the whole seed leads to histograms with large number of peaks, embryo or parts of embryo (axis, radicle or cotyledons) may be dissected from the seed to obtain the 2C peak in highest intensity (Forti et al., 2018). Though, this approach provides precise data, it may be laborious, thus often the whole seeds are used (Hajrudinović et al., 2015; Matzk et al., 2000).

The measurements of fern spores from herbarium also proved to be very useful, as the spores of Ophioglossacae were able to give results even after 26 years of preservation (Kuo et al., 2021). However, the quality of the analysis and the quantity of measured nuclei decreases over time, as in the previous case (Tang et al., 2023).

2 Aims of the thesis

As flow cytometry is crucial in the field of botany, the knowledge of the factors influencing the measurements in the complex arrangement of flow cytometer is highly important. However, mostly fresh samples are used for this purpose and studies focusing on storage of the plants for flow cytometry via herbarium or silica desiccation mostly focus on angiosperms(Bainard, et al., 2011a; Šmarda & Stančík, 2006; Suda & Trávníček, 2006). Thus, this thesis focuses on the factors influencing the genome size stability and quality of flow cytometry histograms for both samples stored for up to ten years in a silica archive and samples collected in field under controlled conditions, whereas the aims of the thesis are:

- Compilation of literary review focusing on the topic of the thesis
- Determination of maximum storage time for the samples stored in a silica archive
- Assessment of genome size stability and histograms quality of samples stored for ten years in a silica archive
- Measurement of own collected samples using flow cytometry and assessment of their genome size stability and quality of their flow cytometry histograms over time in different storage conditions

3 Materials and methods

3.1 Samples from fern genome silica archive

The first part of the study consisted of an analysis of the maximum time that different species are measurable by flow cytometry (FCM) from material dried in silica gel. Samples were taken from Ferns and lycophytes genome databank and silica archive (FerDA) (Ekrt & Vejvodová, unpublished, depon. in.: Department of Botany, University of South Bohemia, České Budějovice) stored at room temperature. In total 10 species of ferns and lycophytes were selected to this purpose. From those 9 species were measured to supplement the data of already analysed samples, and one species was analysed from already completed analyses (Ekrt & Vejvodová, unpubl.) (Table 2). Time of storages for all the stored samples in FerDA were calculated in winter 2023 and samples for the FCM analyses were selected in descending order of time of storage. For the measurements, usually 3 samples per each year of storage were analysed.

Table 2: Summary of the fern and lycophyte species analysed from FerDA by FCM with indication of usage of measurement data (M) or provided data (D), number of samples analysed per taxon.

Taxon	**Data source**	**Number of supplementary analyses**
Athyrium filix-femina	M	7
Cystopteris fragilis	M	6
Dryopteris affinis	M	12
Dryopteris dilatata	M	6
Dryopteris filix-mas	M	11
Equisetum arvense	M	3
Lycopodium annotinum	D	0
Polystichum aculeatum	M	6
Pteridium aquilinum	M	8
Selaginella selaginoides	M	14

3.2 Plant material sampling and preservation

Another part of this thesis included the experimental attitude to the storage and preservation of selected ferns and lycophytes collected in field. Samples for this part of study were newly collected and dried. For this, 14 fern and lycophyte species were selected (Table 3). Usually, 3 plants were collected per species from different locations. All the plant samples were stored in a wet sealable plastic bag in a refrigerator at 8 °C. Sampling was done within a short time frame at the turn of October and November 2022 under similar conditions (see supplementary data) in Czech Republic, Austria and partially from garden cultivations of L. Ekrt in Telč (Czech Republic).

Each sample was within few days further divided into 3 samples each stored under different conditions. Two of those were mostly leaves, pinnae, or stems with microphylls in the case of lycophyte species, which were dried for 2 weeks at room temperature (RT) over silica. After two weeks, one of those parts was further stored at RT and the other was stored in a freezer at –20 °C. The rest of the plants were dried in a drying chamber (appr. 30°C) and preserved as an herbarium voucher.

3.3 Flow cytometry

For the FCM measurements the Otto I and Otto II buffers were prepared for nuclei isolation. Otto I buffer was prepared from citrate monohydrate (Penta; purity 99.9 %; final concentration 0.1 M) and Tween 20 (Sigma-Aldrich; 10 % v/v solution; final concentration 1% v/v). Otto II buffer was prepared from sodium hydrogen phosphate (Penta; purity 99.9 %; final concentration 0.4 M).

For the DAPI FCM sample analysis approximately 0.5–1 cm^2 of the living sample leaves or 2-10 cm^2 of the desiccated sample leaves were first chopped with the corresponding internal standard in ice cold Otto I buffer with a clean razor (Astra ®). Subsequently, the resulting crude nuclear suspension was filtered over a 42 µm nylon filter to a plastic test tube. After 1 min of incubation the filtered suspension was treated with Otto II buffer containing 2-mercaptoetahnol (Sigma-Aldrich, purity 99.9 %; 2 mg mL^{-1}) and DAPI dye (4,6-diamidino-2-phenylindole; Sigma-Aldrich; 99.9 %; final concentration 4 mg mL^{-1}). After approximately 5 min of incubation with the fluorescent dye, the nuclear suspension was analysed with CyFlow Space flow cytometer (Sysmex) equipped with UV-LED laser (365 nm). The flow rate was set to 1.0 µL s^{-1} and the fluorescence intensity of 3000 particles was recorded.

For the analyses of the sample from silica archive stored for longer periods of time, samples from FerDA required to supplement provided data of previous survey (Ekrt & Vejvodová, unpubl.) analysed by FCM (Table 2). The measurements were conducted from the youngest to the oldest samples, where at least 3 measurements were conducted per each sample if possible. The measurements were terminated when no data were obtained from the FCM analyses of all three samples in the analysed year.

For the collected samples, FCM measurements were first conducted with living samples. Subsequently, plant parts of each stored type (dried over silica, dried over silica and frozen and herbarium voucher) were measured over the course of one year. The measurement intervals were always conducted after 1, 4, 8 and 12 months of storage. If no FCM signal was obtained for one sample in two successive time intervals, it was considered unmeasurable, and no further analyses were done.

For each taxon, a suitable internal standard and its relative fluorescence position (channel) were selected based on the measurement of the living sample and the experimental experience. The same internal standard and its relative fluorescence position were used in all determinations specifically for each species. In total, four species of internal standards were used, i.e., *Bellis perennis*, *Chlorophytum comosum*, *Pisum sativum* 'Ctirad', and *Vicia faba* 'Inovec' (Table 3).

Table 3: Analysed species listed together with their plant family, the selected internal standard Bellis perennis [B], Chlorophytum comosum [Ch], Pisum sativum 'Ctirad' [P], Vicia faba 'Inovec' [V], number of sampled plants and the ploidy level.

Taxon	Family	Ploidy	Plants	Standard
Asplenium ruta-muraria	Aspleniaceae	*4x*	3	V
Asplenium trichomanes subsp. *quadrivalens*	Aspleniaceae	*4x*	3	V
Athyrium filix-femina	Athyriaceae	*2x*	3	Ch
Cystopteris fragilis	Cystopteridaceae	*4x*	3	V
Dryopteris affinis	Dryopteridaceae	*2x*	2	V
Dryopteris dilatata	Dryopteridaceae	*4x*	3	V
Dryopteris filix-mas	Dryopteridaceae	*4x*	3	Ch
Equisetum arvense	Equisetaceae	*2x*	3	Ch
Gymnocarpium dryopteris	Cystopteridaceae	*4x*	2	Ch

Lycopodium annotinum	Lycopodiaceae	*2x*	3	B
Lycopodium clavatum	Lycopodiaceae	*2x*	2	B
Polypodium vulgare	Polypodiaceae	*4x*	3	Ch
Polystichum aculeatum	Dryopteridaceae	*4x*	3	P
Pteridium aquilinum	Dennstaedtiaceae	*2x*	3	V

3.4 Primary data evaluation

The histograms obtained from FCM analyses were evaluated in Flowing Software 2.5.1 (P. Therho, University of Turku, freeware available at https://bioscience.fi/services/cell-imaging/flowing-software/).

For all samples first the peak positions (G_0/G_1 and eventually G_2) of the sample and of the internal standard were evaluated. Then the ratio of G_0/G_1 peaks of the sample and internal standard were calculated respectively. Here, the peak ratio of the stored samples was compared to the corresponding ratio of the living sample, and their ratio was calculated, while it serves as a measure of the consistency of the genome size estimations under different conditions. In the following chapters this parameter is designated as genome consistency (GK). The coefficient of variation (CV) of all the peaks was determined, as this parameter shows the variance within individual peaks. Finally, the abundance of samples with no obtained signal was determined, which serves as a measure of probability that a sample will be measurable after certain amount of time in certain storage conditions.

For the samples collected in the field and for the supplementary analysis of the FerDA samples, the event counts were recorded for all the peaks, and from those data the overall background noise (BN) of the histogram was calculated according to Equation 4,

$$BN = \frac{E_t - \sum_i E_i}{E_t} \qquad \text{(Equation 4)}$$

where BN is the background noise, E_t is the total events count and E_i is the events count in i^{th} peak. The BN shows how many nuclei remain measurable at the peak position. It is also the measure of the number of nuclei in a peak obtained for an analysis.

3.5 Statistical treatment

The data from the analysis of the fern genome silica archive were evaluated by simple or multiple linear regression for each species and each of the GK, CV and BN parameters. The simple regression was used for the analysis of the dependence of the measured parameters

for individual species, which were measured as a supplementary analysis. Multiple regression was used to study the influence of storage time and number of chromosomes for the whole dataset for all the three parameters.

For the collected samples, the influence of ploidy storage time and storage conditions on the three parameters was evaluated by analysis of variance (ANOVA). The differences among different categories were tested with the post hoc Tukey honestly significant difference (Tukey HSD) test. The variances of the data in the corresponding categories were evaluated with Levene's test, as it is not sensitive to the discrepancies in normality of the data. Furthermore, this test was used for the verification of the assumptions of ANOVA. The influence of the total number of chromosomes and storage time on the parameters was evaluated by multiple linear regression. The abundance of samples with no signal was counted.

4 Results

4.1 Samples from fern genome silica archive

The maximum storage times of the 10 species analysed from FerDA ranged from 1 to 7 years of storage (see Table 4). Here, it can be seen that the species *Selaginella selaginoides* and *Cystopteris fragilis* last measurable up to 6 and 7 years respectively under the silica storage, while other species such as *Equisetum arvense, Dryopteris dilatata* or *Polystichum aculeatum* last only 1 year under such conditions.

Table 4: The maximum storage times for the measurements in DAPI flow cytometry analysis in the genome silica archive for the 10 analysed species.

Taxon	Maximum storage time [years]
Athyrium filix-femina	2
Cystopteris fragilis	7
Dryopteris affinis	4
Dryopteris dilatata	1
Dryopteris filix-mas	4
Equisetum arvense	1
Lycopodium annotinum	5
Polystichum aculeatum	1
Pteridium aquilinum	2
Selaginella selaginoides	6

The coefficients of determination (R^2) together with the probabilities of the null hypothesis of the linear regression were calculated for background noise (BN), coefficient of variation (CV) and genome consistency (GK) for each of the analysed species (Table 5). The species *Cystopteris fragilis*, *Dryopteris dilatata*, *Dryopteris filix-mas*, *Pteridium aquilinum* and *Selaginella selaginoides* show low to moderate dependence for all parameters, while some of the other species have nearly no dependence on time. The BN, compared to other parameters, has the most significant slopes. Some of the parameters for the species with low maximum storage time (1 year) were not evaluable due to insufficient number of determined values (Table 4), so the data were supplemented from the data obtained for collected samples. The data for the BN are missing for *Lycopodium annotinum*

as for this species no supplementary analysis was performed and the background noise was not evaluated in the provided data.

Table 5: The coefficients of determination (R^2) and probabilities of the null hypothesis of the linear regression for the slope, for the dependence of genome consistency, coefficient of variation and background noise on the storage time. The data taken from the collected samples are marked with a cross (†).

Taxon	Genome consistency		Coefficient of variation		Background noise	
	R^2	p	R^2	p	R^2	p
Athyrium filix-femina	0.833	0.031	0.035	0.763	0.516	0.069
Cystopteris fragilis	0.250	0.667	0.999	0.022	0.372	0.1981
Dryopteris affinis	0.102	0.402	0.052	0.554	0209	0.010
Dryopteris dilatata	0.324†	0.053†	0.232†	0.113†	< 0.001	0.953
Dryopteris filix-mas	0.800	0.003	0.489	0.054	0.245	0.006
Equisetum arvense	0.312†	0.059†	0.347†	0.044†	0.345*	0.045*
Lycopodium annotinum	0.021	0.337	0.129	0.014	-	-
Polystichum aculeatum	0.329†	0.065†	0.130†	0.277†	0.037	0.717
Pteridium aquilinum	0.444	0.148	0.022	0.781	0.691	0.011
Selaginella selaginoides	0.344	0.058	0.285	0.091	0.275	0.066

Furthermore, the correlation between the maximum storage time and the slopes (Table 6) of the lines for the linear regression of the influence of background on time of storage was tested. Here, only the slopes of linear regressions with $R^2 > 0.2$ were tested. The Pearson's correlation test showed that the correlation is significant ($p < 0.001$). A linear regression of those two parameters yielded a coefficient of determination $R^2 = 0.892$ (Figure 1). A strong dependence of the slopes for the BN for the individual species on the maximum storage time was observed. Additionally, for the species with the biggest maximum storage time *C. fragilis* (Table 4) a small negative slope was found, while all the other species show positive slopes of the BN over time. However, with decreasing maximum storage time for DAPI FCM measurements the slopes increase.

Table 6: Slopes obtained for the linear regression of the dependence of background on the storage time of FerDA samples.

Taxon	Slope [years^{-1}]
Athyrium filix-femina	0.096
Cystopteris fragilis	-0.070
Dryopteris affinis	0.038
Dryopteris filix-mas	0.083
Pteridium aquilinum	0.191
Selaginella selaginoides	0.018
Equisetum arvense	0.141

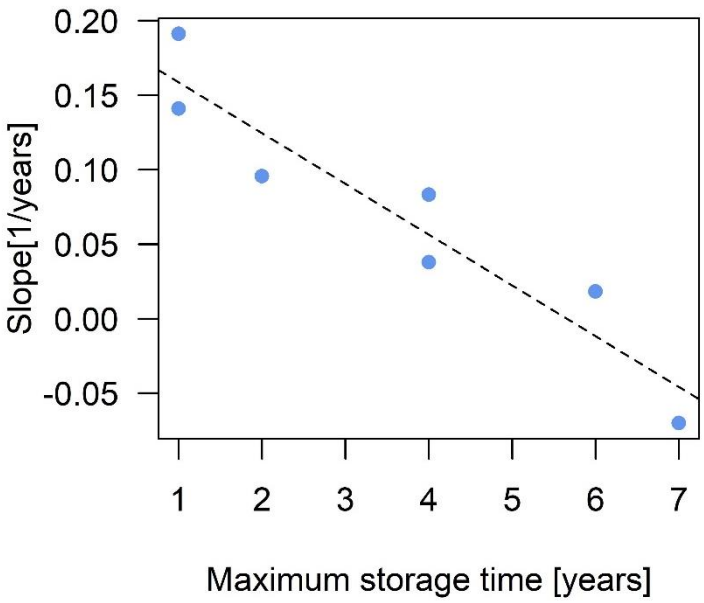

Figure 1: Linear regression of the dependence of the average slope of the background increase in individual species on maximum storage time.

The same correlation was tested also for the coefficient of variation and GK, though there was no significant correlation for those two parameters with $p = 0.635$ for CV and $p = 0.810$ for GK.

Finally, the influence of the number of chromosomes on the GK, CV, and BN was also tested together with storage time in a multiple regression analysis. All the predictors show significant slopes and low to moderate dependence for all three evaluated parameters (Table 7). The plots

representing the regression analysis (Figure 2) show that except for BN the slopes increase with the number of chromosomes. Additionally, the biggest slope was found for the GK in the direction of chromosomes, while GK showed the lowest slope in the direction of storage time.

Table 7: The probabilities of the null hypothesis of linear regression for number of chromosomes and storage time together with coefficient of determination. All values are shown for background noise (BN), coefficient of variation (CV) and genome consistency (GK).

Parameters	$p_{chromosomes}$	$p_{storage\ time}$	R^2
BN	0.0124	<0.001	0.2902
CV	<0.001	0.028	0.4344
GK	<0.001	0.0421	0.5885

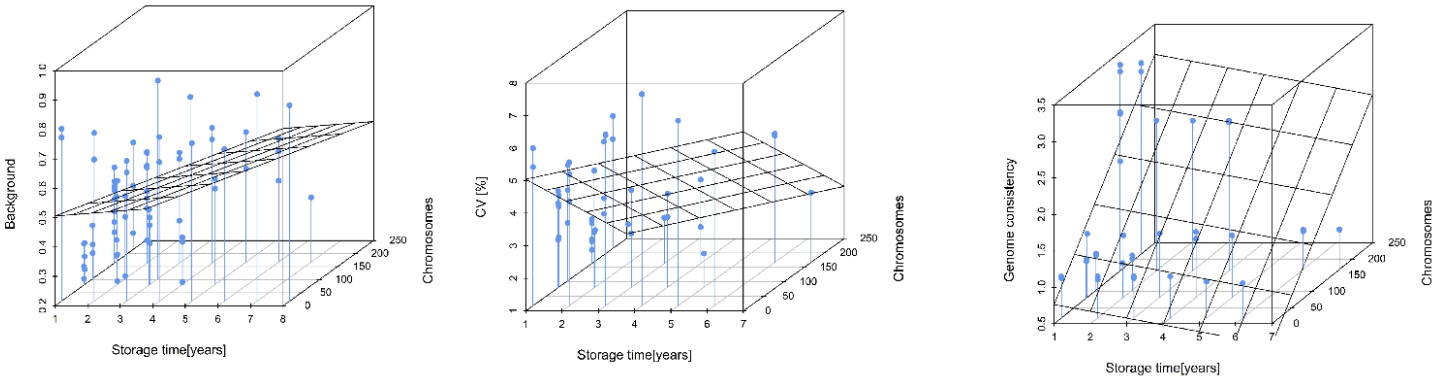

Figure 2: Regression planes for the multiple regression analysis of the dependence of BN (left), CV (middle) and GK (right) on the storage time and number of chromosomes.

4.2 Collected samples

The abundance of samples for which no signal was obtained range from 1 to 9 samples (Table 8). It is evident that the herbarium storage condition has the biggest abundance of the samples with no signals in time. A huge increase in the number of such samples is present even after one month of storage (Figure 3). Both silica gel storage conditions contain only the species *G. dryopteris* which is also the most abundant species among the samples with no signals with no signals with *E. arvense*.

Table 8: Abundance of samples for which no signal was obtained for the individual fern and lycophyte species. The samples are ordered in the descending number of non-measurable samples. The left part of the table shows the species with huge counts of such samples, right part shows the species with rather random occurrence.

Taxon	Abundance	Taxon	Abundance
Gymnocarpium dryopteris	9	*Athyrium filix-femina*	2
Equisetum arvense	9	*Polystichum aculeatum*	2
Pteridium aquilinum	7	*Dryopteris dilatata*	1

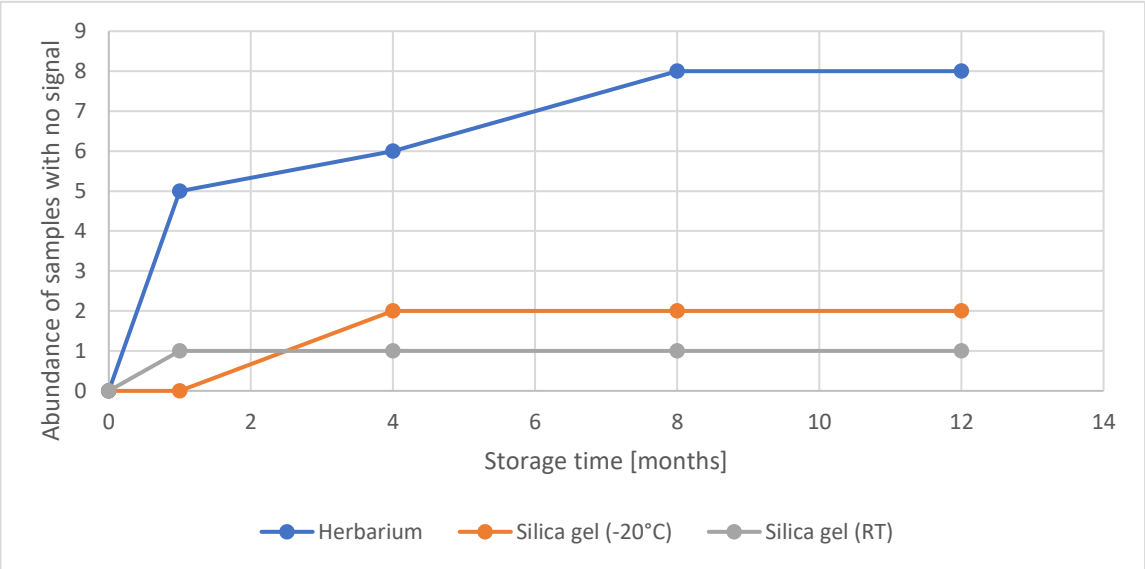

Figure 3: Cumulative curve showing the abundance of samples for which no signal was obtained for different storage types.

Most of ANOVA analyses showed a significant difference for ploidy, storage times, and storage conditions for all the parameters (BN, CV, GK) except for the difference in GK for ploidy and except for the difference in CV for storage conditions where there was no significant difference (Table 9; Figure 4–8). The samples with no signal were analysed for BN, though they were not analysed for CV and GK.

As ploidy contains only two groups (*2x*, *4x*), the significant difference in ANOVA means directly a significant difference between diploids and tetraploids. There are significant

differences for BN and CV, but there is no difference for the GK (Figure 4). The BN was bigger for tetraploids, while for the CV the diploids showed larger values than tetraploids.

For storage conditions, there is a significant difference between the living samples and all the other storage conditions in BN, however, the storage conditions are comparable among each other, whereas living samples have lower BN than all the other storage conditions. For the CV, there is a significant difference between the living samples with lower CV and the silica gel desiccation at room temperature with higher CV. The two left storage conditions are comparable among each other, whereas their CV is between the CV of the living samples and samples stored with silica gel at room temperature. Only herbarium vouchers differ for GK with slightly increased values, whereas the left storage conditions are comparable among each other (Figure 5)

The time of storage aspect was not subjected to Tukey HSD test and the possible differences and trends were assessed only graphically.

Table 9: The probability of null hypothesis of ANOVA (p) together with the F value (F) and the degrees of freedom (df) for the influence of ploidy, storage time and storage conditions on background noise (BN), coefficient of variation (CV) and genome consistency (GK).

Parameters	**Ploidy**			**Storage time**			**Storage conditions**		
	p	F	df	p	F	df	p	F	df
BN	<0.001	63.084	1	<0.001	30.340	4	0.039	3.279	2
CV	<0.001	47.959	1	0.001	4.600	4	0.112	2.199	2
GK	0.122	2.407	1	<0.001	11.891	4	0.001	4.622	2

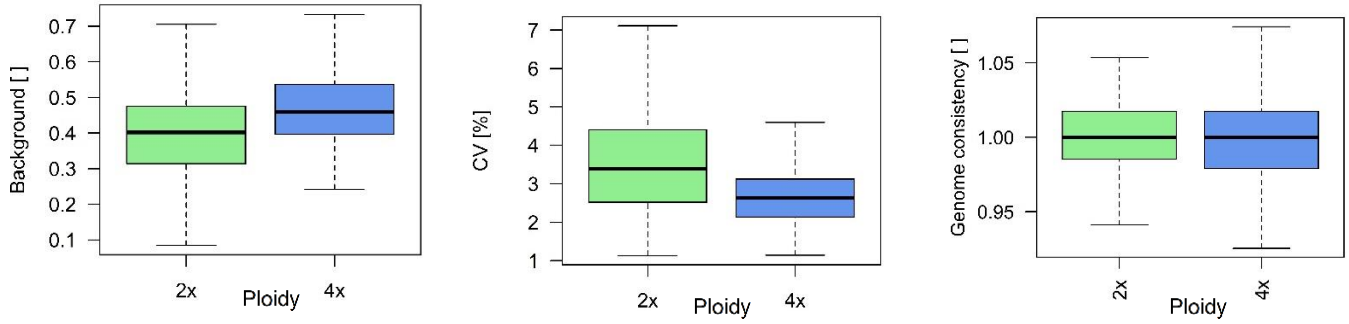

Figure 4: Boxplots showing the differences between diploid (2x) and tetraploid (4x) species for the three studied parameters for the whole dataset.

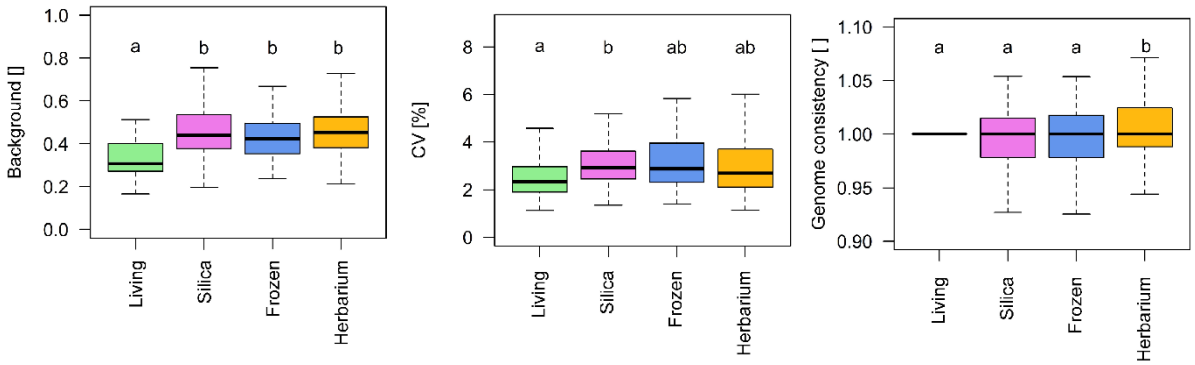

Figure 5: Boxplots showing the influence of the storage type (fresh samples = Living, silica gel at RT = Silica, silica gel at -20°C = Frozen, herbarium = Herbarium) for the backgournd noise (left), coefficient of variation (CV; midle) and genome consistency (right) for the whole dataset, letters denote the results of Tukey HSD test.

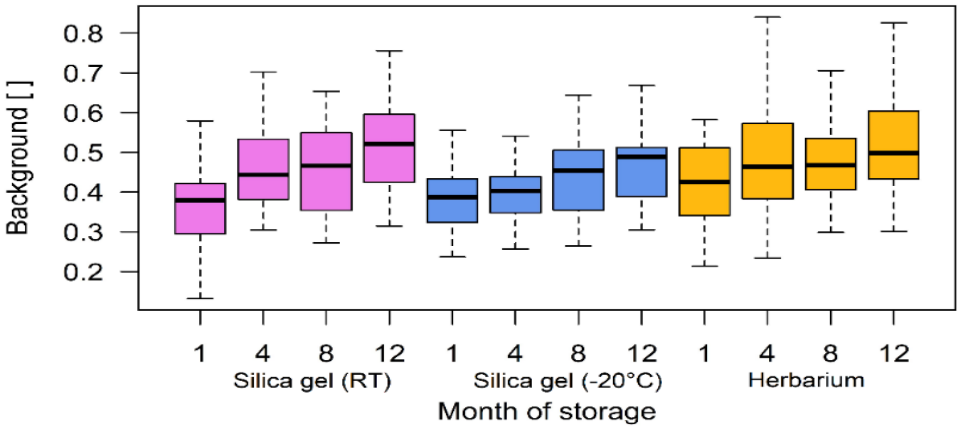

Figure 6: Boxplot showing the influence of time of storage and storage type on the background noise.

It is evident that the BN for all three storage conditions increases with the time (Figure 6). Even though after 1 month of storage most of the samples show similar BN levels, after 4 and more months the BN of silica gel at room temperature is higher than for the other two storage conditions.

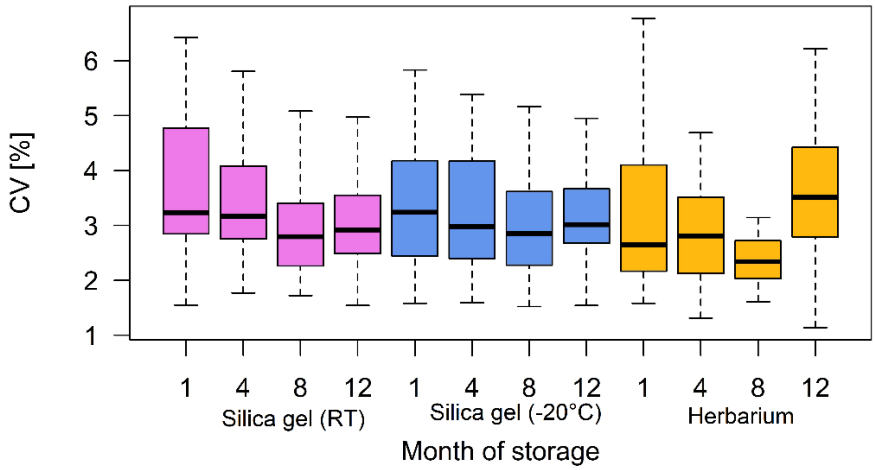

Figure 7: Boxplot showing the influence of time of storage and storage type on the coefficient of variation (CV).

In general, a slight decrease of CV over time for all three storage conditions is obvious (Figure 7). For all of them there is a slight (silica gel storage at RT and silica gel storage at - 20 °C) or huge (herbarium storage) increase in the CV for the last measurement. The difference among storage conditions is not significant (Figure 5).

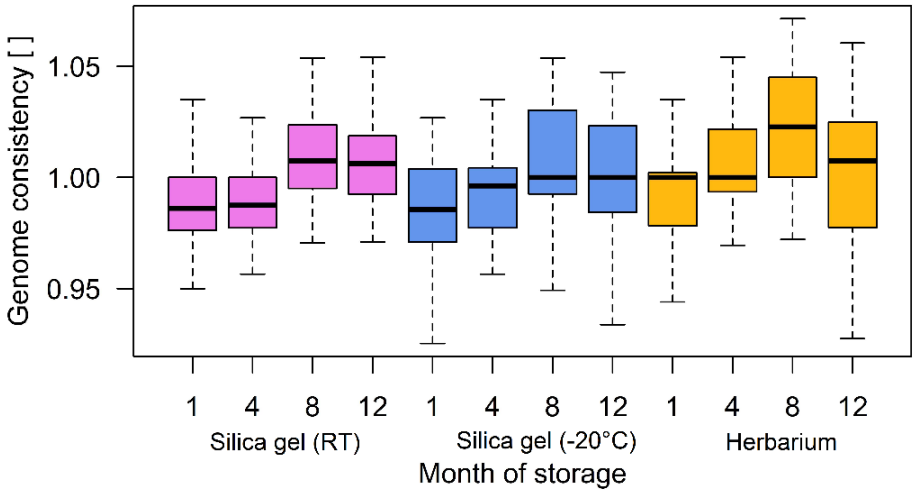

Figure 8: Boxplot showing the influence of time of storage and storage type on the genome consistency.

The ratio of the fluorescence of the sample and the internal standard is slightly decreased for the first two measurements points (for month zero GK is 1) and after 8 months the GK is increased (Figure 8). However, the different storage conditions do not differ significantly.

Most of the results of Levene's test for the variance difference were insignificant (Table 10). Here, the storage type, the time of storage (as time points) and ploidy was evaluated for BN, CV, and GK

Table 10: The results of the Levene's test for the comparison of the variances of the obtained data. The probability of the null hypothesis (p) together with the F value (F) and degrees of freedom (df) is shown for all the predictors (storage conditions, storage time and ploidy) and all the evaluated parameters.

Parameters	Storage conditions			Storage time			Ploidy		
	p	F	df	p	F	df	p	F	df
BN	0.1053	2.0549	3	0.2075	1.4781	4	0.0051	7.897	1
CV	0.2217	1.4710	3	0.0284	2.7381	4	<0.001	17.805	1
GK	0.8203	0.1982	3	0.0279	3.0653	4	0.5581	0.3435	1

The insignificance of most of the results of the Levene's test for the comparison of variances (Table 10) justifies the use of ANOVA for the analysis of the experimental data. The significant results for the storage time for CV are due to the increased variance after the first ($\sigma^2 = 1.320$) and fourth month ($\sigma^2 = 1.116$) of storage and for GK due to the increased variance after the third month of storage ($\sigma^2 = 8.870 \cdot 10^{-4}$). Both significant results for the ploidy are due to the lower variance of the tetraploid samples compared to the diploid samples (Figure 4).

For BN and GK the slopes in the multiple regression analyses of the influence of the number of chromosomes and storage time were significant except for the CV. The CVs showed only a weak trend for the BN and barely any correlation neither for the GK ($R^2 = 0.0755$) nor for CV ($R^2 = 0.0080$) (Table 11; Figure 9).

Table 11: The probabilities of the null hypothesis of linear regression for both chromosome number (chromosomes) and storage time (storage time) together with the coefficient of determination of the linear regression.

Parameters	$p_{chromosomes}$	$p_{storage\ time}$	R^2
BN	<0.001	<0.001	0.2354
CV	0.535	0.227	0.0080
GK	<0.001	<0.001	0.0755

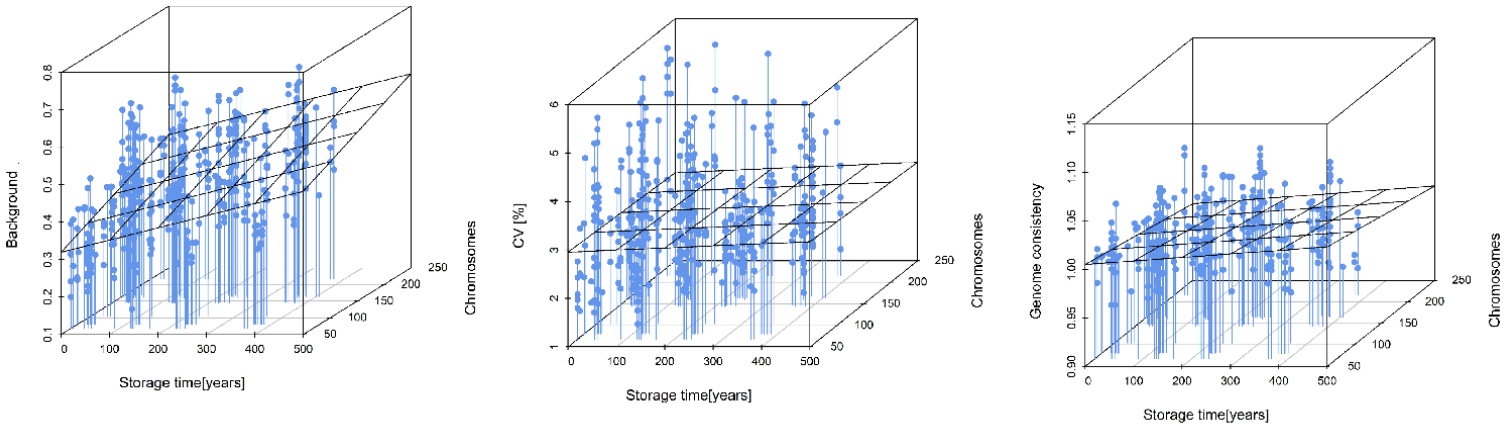

Figure 9: Graphs representing the results of multiple regressions analysis for background noise, coefficient of variation (CV) (middle) and genome consistency (right) without outliers.

Compared to the results of chapter 3.1 (longer time scale comparison), the coefficients of the determination are much lower. Except for the CV there are significant slopes for both analysed factors (storage time number of chromosomes).

5 Discussion

5.1 Samples from FerDA silica archive

One of the goals of the study was to evaluate the storage quality of silica-dried samples in FerDA silica archive in time. This information is crucial for usability of the conserved samples for furhter flow cytometry or molecular studies. The results for individual species are quite contrasting (Table 4–6). The longest maximum storage times for silica storage at room temperature in FerDA were obtained for *Cystopteris fragilis*, *Lycopodium annotinum* and *Selaginella selaginoides*. The two latter species are lycophytes, suggesting, that this most primitive group of vascular plants might have long maximum storage times (Little et al., 2007). Even though some lycophytes are known to resist long periods of drought via poikilohydric mechanisms e.g. *Selaginella lepidophylla* (Pampurova & Van Dijck, 2014), others require quite humid environments (Arrigo et al., 2013). Thus, this might not be the reason of the long maximum storage time under silica gel desiccation. Furthermore, the lycophytes are known to contain significant amounts and different kinds of alkaloids, glycosides and terpenoids (Ma & Gang, 2004; Wang et al., 2022), so the secondary metabolites probably do not play such a role.

Quite a few groups of secondary metabolites are present in *Cystopteris fragilis*, meaning that the amount of different groups of secondary metabolites may be the explanatory factor with respect to maximum storage time (Mir et al., 2014). This is also in compliance with other data obtained by Mir et al. (2014) for *Equisetum arvense* and *Pteridium aquilinum* which also contain more groups of secondary metabolites, and lasted only for 1 year and 2 years respectively. In particular, in regard to secondary metabolites in the present group, the influence of alkaloids and saponins may be significant, as those are present in *E. arvense* and in *P. aquilinum* and not in *C. fragilis* (Table 12) (Čertner et al., 2022; Doležel et al., 2007a). However, for the lycophytes alkaloids and glycosides do not seem to play a significant role, so either the effect is caused mainly by saponins, or there are different mechanisms of the degradation for different pteridophyte groups. Compared to the articles studying angiosperms (Bainard, et al., 2011a; Šmarda & Stančík, 2006; Suda & Trávníček, 2006), some of the pteridophyte species seem to have much longer periods of storage time under desiccation in which they remain measurable with flow cytometry, particularly, the genera *Cystopteris* and *Selaginella*. The latter was measurable for even longer

(for approximately 14 years) in flow cytometry in the study of Little et al. (2007). However, in this study the plant was stored as herbarium vouchers and it was being rehydrated under vacuum for 12 h prior to the measurement. Additionally, Little et al. (2007) analysed a different species (*Sellaginella moellendorffii*), which might already significantly impact the results.

Table 12: The comparison of the metabolite groups detected in the qualitative analysis of secondary metabolites in common species of ferns (Mir et al., 2014*), minus signs (-) denote that the metabolite was not present in the species, plus sign (+) denotes the presence of the metabolite in the species.*

Group of metabolites	***Cystopteris fragilis***	***Equisetum arvense***	***Pteridium aquilinum***	***Gymnocarpium dryopteris***
Alkaloids	-	+	+	+
Glycosides	-	+	-	+
Resins	-	-	+	-
Saponins	-	+	+	+
Phenolics and tannins	+	+	+	+
Volatile oils	+	+	+	+
Terpenoids	+	-	-	+
Flavonoids	+	+	-	+
Phlobatannins	-	-	+	-

The simple regression analysis of the individual species showed that the genome size estimations are mostly consistent with no significant slope. Although some species showed significant slopes, the coefficients of determination for those species are very low meaning that the slope probably resulted from random bigger variation of the experimental data. Similar results were obtained for the coefficient of variation (CV), for which no trend was found. The fact that no trend was found for CV is surprisingly in conflict with studies, as here the CVs increase over time under all storage conditions, but most of the measurements in those studies were conducted on angiosperms (Bainard, et al., 2011a; Suda & Trávníček, 2006)

The simple regression analysis of the background noise (BN) showed that there exists probably a low to moderate trend, as the species with significant slopes also had higher coefficients of determination. Furthermore, the linear regression of the slopes against maximum storage

times suggests that the BN may play an important role in the disappearance of the signal in the flow cytometry analysis. As the BN is a reciprocal measure of the peak heights normalised to the total amount of nuclei in the histogram, this result is also in compliance with the data of Suda and Trávníček (2006), who determined that for all the angiosperms and two pteridophyte species they analysed, the peak height decreased. Additionally, they also observed an increasing background fluorescence for all the storage types.

The analysis of the influence of time and the number of chromosomes showed by multiple linear regression revealed that there is a significant slope for both of those predictors. Furthermore, compared to the simple regression analysis, the coefficients of determinations are higher. The largest R^2 value was obtained for the genome consistency (GK), where the slope for the influence of time is not so significant, although there is a strong influence of the number of chromosomes with a positive slope. This might mean that the stability is either species specific and by chance the species with higher variation in GK were by chance ordered in the increasing number of chromosomes, or rather, that the GK is to some extent dependent on the number of chromosomes present in the nucleus. The finding is not in line with the literature data (Bainard, et al., 2011a; Little et al., 2007; Suda & Trávníček, 2006), where the GK is typically very stable regardless of the species or storage time, though, the significant trend in this work may be influenced by quite low number of measured samples or obtained data. Furthermore, for the FerDA samples, the conditions have been uncontrolled, which could cause the appearance of a false trend. Similar trend was observed for the BN, for which two positive slopes were also determined for both time of storage and number of chromosomes. This result may be supported by the claim of Suda and Trávníček (2006) that samples of higher ploidy have shorter lifetime than their diploid counterparts.

The results for the CV show two negative slopes for both used predictors. This is again in conflict with literature, as the CV typically increases with time for the stored species. This might be caused by the manual evaluation of the FCM histograms, wherein a false positive from the background might have increased the height of the peak in the histogram resulting in the decreased standard deviation of the peak due to higher number of events within the similar range of variance. In turn the decrease of the standard deviation decreases the CV resulting in the observed trend. However, the decrease in CV can be just as well explained by shift of the nuclei previously increasing the CV into the background, where they do not influence the quality of the peak anymore except for the decrease in events in the peak.

The obvious limitation which we were aware of for the samples stored in FerDA is that the circumstances of the samples collection and preparation is not standardized through time, as the archive contains samples collected in different years, by different collectors, with different times between collection and silica gel storage, the year of storage and possibly different ecological conditions in which the plants grew (altitude, habitat type, moisture, shading etc.). Furthermore, as the analysis were conducted in the manner so that time influence could be studied, the number of analysed samples is rather low. This brings an uncertainty to the results, which may also be the reason for the huge coefficients of determination for the individual species. For more precise results further research should be conducted, whereas more samples should be collected each year either at the same time of the year and at similar time and similar environmental conditions.

5.2 Collected samples

The abundance of samples with no signal is the highest for the species *G. dryopteris, E. arvense* and *P. aquilinum*. This is again in compliance with the data obtained for FerDA, where the two latter species remained under storage only 1 and 2 years respectively. Furthermore, Mir et al. (2014) also found a high level of the secondary metabolites not only for *E. arvense* and *P. aquilinum* but also for *G. dryopteris*, particularly alkaloids, glycosides and saponins (Table 12). Most of the samples with no obtained signal belonged to the herbarium stored samples. This means that storage in herbarium can not only shift the fluorescence peak significantly from the original position, which might lead to incorrect results in the analysis, but that there is also a higher probability of losing the sample signal completely.

The samples, particularly those of *G. dryopteris*, which were one measurement before the complete disappearance of the signal, were a huge source of outliers in the analysis. Those outliers were present mostly for the GK parameter, whereas the BN and CV remained constant. Those results are in compliance with the data obtained for other groups of plants (Bainard, et al., 2011a; Šmarda, 2008; Šmarda & Stančík, 2006; Suda & Trávníček, 2006).

For the experimental part of the thesis, 39 fresh samples of ferns and lycophytes were collected and evaluated under standardized conditions (Table 3). ANOVA tests for ploidy revealed that there is a significant difference for BN and for the CV, however, there was no significant difference found for the GK. For BN the tetraploid samples showed higher level of the BN than diploid samples. This might be due to the fact that for the tetraploid samples

the probability of obtaining different fluorescence is higher, as there are more copies of the DNA, thus it should be more sensitive to fluorescence inhibitors. Additionally, a higher fluorescence might also be possible, as there might be fluorescent debris, or the nuclei might be fused together (Doležel et al., 2007a; Loureiro et al., 2021). This proposed mechanism may also explain the values obtained for the CV. Here the diploid samples have higher CV than the tetraploid, which may be explained by the nuclear DNA content. While the tetraploid nuclei are sensitive and are probably more shifted to the background, the diploid nuclei are probably shifted more within the range of the peak, thus increasing the CV. The shift of the whole peak on the x-axis is not significant, meaning that there is no significant difference in the variation of the genome size estimation for diploids and tetraploids.

The influence of the storage on the BN significant, however there is only a significant difference among the living samples with lower BN and the other storage conditions with higher BN. Among the individual storage conditions there is no significant difference, which was revealed with the Tukey HSD test. Similar results were obtained for the CV, whereas there is a slight difference between the samples stored under silica gel desiccation at room temperature, and the two other storage conditions (silica gel desiccation at -20°C and herbarium vouchers). For the GK, significantly higher values were obtained for the herbarium vouchers, while for the silica gel storage at both temperature values comparable with the living samples were obtained. This means that for all the parameters the traditionally used herbarium vouchers are comparable or even slightly worse than the silica gel desiccation storage conditions for ferns and lycophytes. Similar results were obtained for mostly angiosperms and two species of ferns by Suda and Trávníček (2006), who showed that silica gel desiccation is comparable to herbarium desiccation in flow cytometry analysis. Furthermore, the results for CV suggest that it is probably beneficial to store the silica gel desiccated samples under decreased temperature even if deep freezing is not possible, as it was previously proposed by Suda and Trávníček (2006) for the angiosperm plants.

The influence of time was significant for all the studied parameters. While BN and the genome size mostly increased over time, CV decreased. The decrease in CV is in contradiction with the data obtained by similar studies for mainly angiosperm groups (Bainard, et al., 2011a; Doležel et al., 2007a; Suda & Trávníček, 2006). This might mean that within ferns and lycophytes a bigger concentration of the coatings of fluorescent debris (Doležel et al., 2007a; Loureiro et al., 2021) is present, meaning that the nuclei are shifted from the peak into the BN,

thus further decreasing the CV, while increasing the BN of the histograms. This is further supported by the data obtained for GK, as the genome size increases over time for all the storage conditions.

The Levene's test for the differences between variances of the storage types was conducted and showed that mostly there is little or no significant difference among the different studied parameters. All the parameters for the storage conditions are insignificant, meaning that the variance for all BN, CV and GK are the same for all the storage conditions. For the storage time there is a significant difference in CV and GK. For both CV and GK there is an increased variance in time. This means that with time there is a higher probability of obtaining data with worse quality or incorrect genome size. This was also claimed by several other studies and publications focusing on the measurement of dehydrated tissues of mostly angiosperms with flow cytometry, whereas those studies report higher backgrounds, shifts in fluorescence or signal disappearance (Bainard, et al., 2011a; Doležel et al., 2007a; Suda & Trávníček, 2006).

For the ploidy a significant difference was shown for the BN and CV The results for the BN are in compliance with the data obtained by Suda and Trávníček (2006), who showed that polyploid plants have shorter lifetimes than their diploid counterparts. Nevertheless, CV, which was higher for diploids, showed opposite results compared to study of Suda and Trávníček (2006). Both of those results may be explained by higher accumulation of secondary metabolites in tetraploids (Lavania et al., 2012; Park et al., 2021), as those metabolites may cause the shift of some nuclei from the peak to the background. Thus, the CV of the peak is decreased, however, the background is increased for tetraploids.

Finally, the influence of the number of chromosomes and the storage time was tested. Except for the CV, the slopes for both storage times and number of chromosomes were significant. However, only a weak coefficient of determination was obtained for the BN and almost no coefficient of determination was obtained for the GK. This means that for the timeframe of one year there is a weak, though quite consistent trend for the background, however the GK is a subject of a rather random variation. The increase of the background is in compliance with the studies conducted on the similar topic for other plant groups (Bainard, et al., 2011a; Doležel et al., 2007a; Loureiro et al., 2021; Suda & Trávníček, 2006). The CV seems not to change significantly either with the number of chromosomes nor with the time of storage under desiccation.

The important limitation of this experimental part of the thesis is the instrument itself. Even though only one flow cytometrical device Sysmex CyFlow was used. This device is used quite intensively for a variety of purposes, especially in spring and summer, which could potentially vary the quality of the measurements slightly. Ideally, such measurements should be conducted on an instrument which is extensively cleaned, and used only for this purpose. This is, of course, an ideal situation that cannot be practically achieved. Furthermore, despite the fact the internal standards are mostly and collected at their best conditions, they possess a certain level of their own variability. Even though the standards were measured individually before each set of analysis, they still may be influenced by some other factors within the analysis, such as the secondary metabolites in the ferns. However, if the secondary metabolites would lead to similar shift for both the sample and the standard, the resulting ratio of the sample to standard fluorescence should remain very similar.

6 Conclusion

For the analysis of the ferns and lycophytes genome silica archive FerDA it was shown that:

- In time the coefficient of variation and the genome size do not vary significantly.
- Background noise of the flow cytometry histograms shows weak to moderate increasing trend for individual species.
- Higher number of chromosomes and long storage time periods have negative significant influence on the quality of the flow cytometry histograms.

For the experimentally collected samples it was shown that:

- Storage conditions generally do not influence the quality of the flow cytometry histograms in time for ferns and lycophytes.
- Herbarium vouchers have an increased risk of the sample signal disappearance in the flow cytometry histograms.
- Higher number of chromosomes and long storage time periods significantly increase the background noise of the flow cytometry histograms.
- The background noise is significantly higher for tetraploid plants, while coefficient of variation is significantly higher for diploid plants in flow cytometry histograms.

7 List of used literature

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