High mountains of Central Europe as a refuge of surprising cytotype diversity of Huperzia selago (Lycopodiaceae)

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Abstract

Polyploidization is pivotal in plant speciation, enhancing adaptability, ecological tolerance and specific geographical distribution pattern. While cytotype diversity is extensively studied in angiosperms and ferns, knowledge in homosporous lycophytes remains very limited. This study addresses this gap, focusing on the homosporous lycophyte *Huperzia selago* in Central Europe. Employing flow cytometry, we assessed genome size in 1330 *H. selago* individuals from 208 populations identifying five distinctive cytotypes (2*x*, 3*x*, 4*x*, 5*x*, 6*x*). Challenging chromosome counting on somatic gemmae roots was applied for the three lowest cytotypes yielded average counts of 140, 204, and 262 chromosomes, respectively. Geographical separation of cytotypes was not observed. Tetraploids were most widespread (72.7%), while triploids (21.3%) were rare, and extremely rare are cytotypes 2*x*, 5*x*, 6x constituted less than 5% of the dataset. Uncommon cytotypes were associated with the Alps and triploids occurs also in the highest parts of Western Carpathians. Hexaploid genome size (29 pg) approached upper limits reported in lycophytes. Around 27.3% of populations exhibited mixed cytotypes. Except for atypical diploids, spore abortion differed between even and odd ploidies, approximately 21.8% and 65.2%, respectively. Microcharacter sizes (stoma, spore) did not linearly correspond with increasing ploidy levels. The high ploidy-level diversity and cytotype coexistence in Central European *Huperzia selago* match the well documented patterns in ferns and angiosperms. These findings provide valuable insights into lycophyte polyploidy, underscoring the necessity for broader geographical sampling and applicance of molecular studies to elucidate phylogenetic relationships and taxonomic classifications within the genus *Huperzia*.

INTRODUCTION

Polyploidization is one of the main mechanisms of the plant speciation process. This phenomenon has been estimated in 15% of flowering plants and 31% of homosporous fern speciation events (Wood et al. 2009). Polyploidy confers several advantages, including the restoration of fertility even when different parental genomes are involved, increased gene diversity linked to the attenuation recessive allele effects by dominant types, alteration in gene expression leading to novel adaptations, and facilitation of selfing and asexual reproduction (Comai 2005). Consequently, polyploids are known to exhibit greater ecological tolerance (Ramsey 2011), adapt more readily to harsh and colder environmental conditions (Kirchheimer et al. 2016), demonstrate enhanced potential for evolutionary novelty and changes in reproductive mechanisms, and display increased reproductive success under suboptimal conditions (te Beest et al. 2012). Conversely, higher ploidy levels may pose challenges such as the risk of aneuploidy due to changes in cellular structure and errors during meiosis (Comai 2005), as well as "unintended" gene silencing, where targeted active alleles can be silenced (Mittelsten Scheid et al. 2003).

Changes in ploidy level may be caused by species fluctuations driven by climatic changes, secondary contact of isolated lineages (Hewitt 2004) or due to cold-induced increased production of unreduced gametes (Brochmann et al. 2004). The Pleistocene alternation of glacial and interglacial periods, causing repeatedly lineages isolation of warm adapted flora and spreading of cold adapted species, played a pivotal role in the evolution of many plant species (Hewitt 2004). As species retreated to lowland areas due to cooling, disjunctive ranges emerged, leading to allopatric speciation (Boucher et al. 2016). Additionally, polyploidization can be commonly associated with sympatric speciation, where ecological speciation and the formation of reproductive barriers take place (Ostevik et al. 2012).

However, both reproductive and geographic barriers between plants of different ploidy levels may not be absolute, with their intensity varying between species and cytotypes (Sonnleitner et al. 2013). Consequently, cytotypic variation can persist even in mixed populations without clear evolutionary significance (Hanušová et al. 2019).

While these mechanisms of speciation are well-studied in angiosperms and ferns, they remain understudied in lycophytes, the earliest diverging vascular plant group. Nevertheless, polyploidization is the source of cytotype

variability. In homosporous lycophyte it have been studied sporadically, e.g. in genus *Lycopodium* (Takamiya and Tanaka 1982), and *Huperzia* (Beitel and Wagner 1982; Takamiya and Kurita 1983). In contrast, in the heterosporous genus *Isoëtes* polyploidy is a common phenomenon. And it is connected with varying degrees of fertility associated with different degrees of meiosis irregularities and aneuploidy across taxa (Suissa et al. 2022). Additionally, in *Isoëtes*, there are indications that polyploidy played a key role in allopatric speciation of new species from isolated lineages (Liu et al. 2004). In Europe, a similar example involves the diversified heterosporous lineages of the *Isoëtes lacustris* complex, where recently diversified species have been recognized based on chromosome counts and distinctive morphological characters (Brunton et al. 2020).

Our study deals with polyploidy in lycophytes of Central Europe, with a particular focus on the homosporous genus *Huperzia*. We extensively examined polyploidy in the relatively common mountain lycophyte, *H. selago* (L.) Schrank and Mart., the sole species of the genus reported thus far in Central Europe. This circumboreal taxon occurs in a very wide range of habitats including mountain forest understory, subalpine and alpine meadows, moist rocks, stony screes and *Pinus mugo* scrubs (Kaplan et al. 2019). Its chromosome number is reported to be 2n = 264 (Manton 1950). In contrast, from northern Europe *H. arctica* (Tolm.) Sipliv is reported. It is a circumpolar taxon growing in tundra and alpine meadows (Blockeel 2006) and is believed to have a chromosome number of 2n = 90 (Sorsa 1962, Valentine and Moore 1993; Blockeel 2006). Furthermore, several other taxa were recently reported from the northern Europe such as *H. continentalis* Testo, A. Haines & A. V. Gilman (Testo et al. 2016), H. *europaea* Björk, and *H. acicularis* Björk (Björk 2020). Their distribution, habitat requirements and ploidy level are not sufficiently known as they have not been recognized until recently. In Europe, the genus also includes the Macaronesian endemic species *H. suberecta* (Lowe) Tardieu, reported from Madeira and the Azores (Blockeel 2006).

In the genus *Huperzia*, various chromosome numbers have been reported for several Asian species: 2n = 134, 136, 204, 264, 272, 276 and 528 (Ghatak 1965, Beitel and Wagner 1982; Takamiya and Kurita 1983; Takamiya 1984; Li et al. 2024), highlighting significant cytotype variability within the genus. Notably, conflicting chromosome counts have been published for *H. selago*: 2*n* = 68 (Hagerup in Hagerup and Peterson 1960, Löve and Löve 1961), 2*n* = 88 (Harmsen in Löve and Löve 1948), 2*n* = 90 (Sorsa 1962), and 2*n* = 264 (Manton 1950). However, some values were later deemed mistaken, correlating with the chromosome number of *Lycopodium* in the same location or were published as single numbers without proper documentation (Löve and Löve 1965). Studying this material is challenging, as indicated in the literature (Manton 1950; Wagner 1992), and chromosome counts from Europe are therefore relatively scarce.

As an alternative to chromosome counting, flow cytometry is nowadays the most widely used method for detecting ploidy levels and genome size (e.g., Sliwinska et al. 2005). However, common practice requires fresh tissues to be used. In contrast, some studies report the successful use of dried tissues of angiosperms (Tomaszewska et al. 2021), ferns (Wolf et al. 2015), mosses (Bainard et al. 2020), and lycophytes (Little et al. 2007; Bainard et al. 2011b). However, the results obtained from dried tissues should be limited to the ploidy level estimation, interpreting fine (within-ploidy) genome size differences is not recommended at all (Sliwinska et al. 2022). Most of these successful measurements were done from silica gel-dried tissues, but air-dried samples (mosses; Bainard et al. 2020) and herbarium vouchers were also successfully measured, including lycophytes (*Selaginella*; Little et al. 2007; Bainard et al. 2011b). We adopted a comprehensive approach in our study, comparing fresh, silica-dried, and air-dried (herbarium) *H. selago* specimens. This allows us to assess the applicability of DNA ploidy level estimation in study materials lacking fresh tissues, such as those from remote areas or existing herbarium specimens.

In samples for which flow cytometry is not feasible or possible (old material, the lab not available to a researcher), traditionally accepted micromorphological characters such as spore abortion, spore size and stomata size, which

often correlate with genome size and ploidy, can be employed (Wagner et al. 1986; Ekrt et al. 2021). However, the validity of these characteristics should be tested via calibration.

In this study, we focused on the cytotype diversity and distribution patterns of the widespread mountain lycopod *Huperzia selago* in broader area of Central Europe. We attempt to answer the following questions: (1) What is the cytotype variation in *H. selago* in Central Europe? (2) Can individual cytotypes be identified as ploidy levels, corresponding with chromosome counts? (3) Do genome size, stoma size, spore size and spore abortion allow determination of the ploidies? (4) Is estimation of genome size from dried material (silica-dried, herbarium vouchers) reliable?

MATERIALS AND METHODS

Field sampling

Plant material was sampled during 2016–2023. The sampling covered all habitats (forests, spring areas, alpine grasslands, rocks, screes, etc.) occupied by the *H. selago* across Central Europe with particular emphasis on Central European mountains. Depending on the plant's abundance at a given locality, we sampled 1–20 randomly selected plants about 2 m apart. Each site was characterized by geographical coordinates, habitat and elevation (see Supplementary Data SI1). Whole plants or a subset of shoots per plant were collected to make herbarium voucher and for flow cytometry (FCM). Samples for FCM were either kept fresh until the analysis or ca 4 cm long part of one shoot was desiccated using silica gel within a day. Herbarium vouchers are deposited in CBFS. Distribution of samples and discovered ploidies and occurrence of the ploidies on elevation scale were displayed in ArcGIS Pro 3.0.3 (ESRI, Los Angeles, USA).

Flow cytometry

Flow cytometry was applied for ploidy identification and genome size estimation in the *H. selago*. We used two-step protocol with Otto buffers (Otto 1992) following the best practice recommendations (Sliwinska et al. 2022). All individual plants or pooled samples usually of two individuals were chopped with the internal standard (amount of the material about 5:4 as sample:standard) in 400 µl of Otto I buffer using a sharp razor blade in a Petri dish and filtered through 42 µm nylon mesh. After 3–5 min 800 µl of Otto II buffer with 2-mercaptoethanol (2 mg/ml) and fluorochrome DAPI (4,6-diamidino-2-phenylindole, final concentration 4 µl/ml) was added into the sample. Stained samples were analysed using a CyFlowSpace instrument (Sysmex-Partec) equipped with 365 nm UV-LED as a light source. Fluorescence intensity of 3000 particles was recorded. *Vicia faba* 'Inovec' (2C DNA = 26.90 pg; Doležel et al. 1992) was used as an internal standard for all measurements. Flow cytometry histograms were evaluated using Flowing Software 2.5.1 (P. Terho, University of Turku, freeware available at https://bioscience.fi/services/cell-imaging/flowing-software/). The relationship between sample relative fluorescence (i.e., ratio of sample/standard mean fluorescence) and ploidy level was calibrated using chromosome counting (see below).

As DAPI stain selectively binds to AT bases of DNA, genome size differences may seemingly appear between samples differing strongly in their genomic AT/GC content. Due to possible differences in AT/GC content between the sample and the standard, it is impossible to calculate sample genome size based on DAPI staining. To address this issue, genome size was determined for a selected subset of samples representing all cytotypes using propidium iodide (PI) staining, which stains all bases indiscriminately. The sample preparation was identical, only using PI at final concentration 50 µg/mL as a fluorochrome instead of DAPI. As internal standards were used *Pisum sativum* 'Ctirad' (2C DNA = 9.09 pg; Doležel et al. 1998) for the DAPI-stained measurements, and *Vicia faba* 'Inovec' (2C DNA = 26.90 pg; Doležel et al. 1992) for PI-stained. Measurements with other standards used because of possible overlaps were

recalculated to these standards, based on calibration of all standards against each other. Fluorescence intensity of 5000 particles was analyzed using a CyFlowSpace instrument (Sysmex-Partec) equipped with a green solid-state laser (Cobolt Samba 532 nm, 100 mW). In PI analyses, samples were always processed individually and a mean value of three measurements on different days was used for genome size calculation.

Most samples were measured from live tissue, but silica-dried or herbarium samples were also used. To compare analysis quality using live, silica-dried and herbarium tissue, 51 samples of the two most common cytotypes (3*x* and 4*x*, see below) were measured by DAPI-staining from each type of tissues and ten samples (3*x* a 4*x*) were measured by PI-staining three times from each type of tissue. In every case, standard tissue was live. For calibration purposes, all samples measured by PI-staining were also measured by DAPI-staining. Differences in the genome size and coefficients of variation (CV) of a sample peak (as a common measure of the analysis quality) were compared between types of tissue.

Statistical analyses were performed in R 4.1.2 (available at https://www.R-project.org/, Vienna). Nested ANOVA with sample identity as a random factor was used to compare the relative genome size and sample CV between the tissue types (separately for each cytotype).

Karyology

Plants of three lowest cytotypes were used for chromosome counting: diploids (2*x*) from population ID 108 (Rasen-Antholz, the Alps, Italy), triploids (3*x*) from population ID 102 (Tatranská Lomnica, Vysoké Tatry Mts, Slovakia), tetraploids (4*x*) from populations ID 45 (Uricani, Retezat Mts, Romania) and 117 (Matrei in Osttirol, Hohe Tauern Mts, Austria), see Supplementary Data SI1 for the locality details. Because of very slow growth of *H. selago* plants, instead of roots of mature plants we used gemmae (vegetative propagules) to get fresh roots with cells undergoing mitosis. The gemmae were placed on wet filter paper in a Petri dish and grown in cultivation box in 20°C and 12 h of light. After several days 2–3 mm long roots were collected and put to the 1,4-dichlorbenzene and incubated in darkness at room temperature for 2–3 hours. After pre-treatment, the roots were rinsed in water and placed into a fixative solution 3:1 etanol:acetic acid and the material was stored in -20°C. Further processing followed the modified protocol of Mandáková and Lysak (2016). The roots were washed in distilled water twice for 5 min, then in cold 1× citrate buffer (0.1 M citric acid monohydrate + 0.1 M trisodium citrate dihydrate mixed in 2:3 ratio and 10× diluted) twice for 5 min and then the buffer was changed for an enzymatic mix (0.3% pectolyase, 0.3% cellulase, 0.3% cytohelicase in citrate buffer). The roots were digested in 37°C for 1-1.5 hours. After digestion, the enzyme mix was changed for cold 1 × citrate buffer for at least 15 min. Then each root was placed onto slide, the meristematic tissue was separated, and the rest of material was discarded. The meristem was disintegrated with preparation needle and 20 µl of 60% acetic acid was added. After 2 min, the slide was placed on heating block at 50°C and the suspension was spreading with a preparation needle for 30 s. Then 100 µl of etanol:acetic acid solution was applied on suspension and the liquid was strained from the slide. The dried slide was stored in 4°C. 15 µl of Vectashield with DAPI was added as a staining solution and the preparation was covered with cover slip and fixed with nail polish. The mitotic nuclei were observed under Nikon Eclipse E600 fluorescence microscope equipped with Nikon DS-Qi1Mc camera and the picture was taken with NIS-Elements AR software.

Spore size and abortion

Spore size was measured on ten plants per each cytotype, with the exception of rare diploids and hexaploids, for which five and two plants were analyzed, respectively. Unripe unseparated tetrads were not counted or measured. Furthermore, the number of well-developed regular spores and putative diplospores (henceforth, we mark with this term spores which are spherical and visibly bigger than others), and the number of aborted regular spores and diplospores were evaluated in all measured samples (Fig. 1).

The yield of the *H. selago* spores from herbarium vouchers is usually relatively small, but where possible, spore length ("spore size") of regular spores was measured in the proximal view. On the side with the proximal vertex (with the trilete mark) upwards, spore length is defined as distance the perpendicular to the center of the any distal edge towards the opposite vertex of the imaginary "triangle" was measured (see Fig. 2). If the spore "triangle" was isosceles (instead of equilateral), the height perpendicular to the base was measured. Diplospore size was measured between the most distant points of the shape because of the oval shape of diplospores. Where possible, 20 regular spores and 10 diplospores were measured for each plant. All measurements were done at 400× magnification. Differences in the regular spores and diplospores size between ploidies were tested using one-way ANOVA with Tukey HSD, based on mean values for each individual.

Spore abortion rates (SAI; Hornych and Ekrt 2017) were determined for all plants used for spore size measurement. In total, 500 (in case this was not possible, only 200 or 250) spores were assessed at 100× magnification in 1% acetocarmine solution (Fig. 1), in which aborted spores with differently shaped exospore and without cell content have different color than ones with well-developed contain. The number of regular spores and diplospores was recorded from the whole amount. One-way ANOVA with Tukey HSD test was used to compare the proportion of aborted regular spores and diplospores between the cytotypes; % of the aborted spores was arcsin-transformed.

Stomata size

For this measurement, clear nail polish was first applied to the underside of microphylls and a transparent adhesive tape was used to make imprint, which was transferred to the slide. Stomata were measured at 400× magnification. Ten stomata per plant were measured, and from each stoma one guard cell was measured longitudinally. Ten plants of each cytotype (the same plant as for SAI and spore size measurements) were analysed, but in the case of the rare cytotypes (diploids, hexaploids) only five and for plants were used, respectively. Length of stomata for individual cytotypes was compared by one-way ANOVA with Tukey HSD test; each individual was represented by the mean value.

All spore and stomata measurements were done using Olympus CX31 microscope equipped with Lumenera Infinity 1 camera and QuickPhoto camera 3.2 software (Promicra Program 2020; www.promicra.com).

RESULTS

Genome sizes and karyology

Flow cytometry screening of 1330 live samples from 208 populations from central Europe revealed five different cytotypes (Fig. 3) with no overlapping values of the (relative) genome sizes between the cytotypes and only small variation within the cytotypes (Fig. 4, Table 1). The chromosome numbers of the three lowest cytotypes were $2n = 140$ (mean of the two best chromosome figures being 137 see Figs. 5 and 143 see Supplementary data Fig. S1), ca 204 (only one figure available) and ca 262 (mean of five best figures (Fig. 5), range 253–265 see Supplementary data Fig. S2). These values approach the expected values based on the published $x = 67$ (Wagner 1992), $2n = 134, 201, 268$ for diploids, triploids and tetraploids, respectively. The two other cytotypes were then identified as DNA-5*x* and DNA-6*x* based on the genome sizes. The monoploid genome size (1C*x* value) was similar among all recorded ploidies, with the maximum value in diploid (5.02 pg) and minimum value in triploid (4.50 pg).

Table 1

Ploidy levels of *H. selago* distinguished by flow cytometry parameters - ratio to the standard (*Vicia faba* ´Inovec´) and genome size; and by chromosome numbers. Used values only from live samples (N).

ploidy level	DAPI staining			PI staining			chromosome count
	ratio to the standard (S.D.)	Min-Max	N	2C mean value in pg (S.D.)	1Cx (pg)	N	
2*x*	0.29 (0.01)	0.29–0.31	9	10.03 (0.35)	5.02	4	ca 137–143
3*x*	0.43 (0.01)	0.42–0.45	555	13.49 (0.18)	4.50	12	ca 204
4*x*	0.59 (0.01)	0.56–0.62	1660	19.23 (1.20)	4.81	21	ca 253–265
5*x*	0.74 (0.01)	0.70–0.75	44	23.22 (0.60)	4.64	9	-
6*x*	0.88 (0.01)	0.86–0.90	14	28.96 (0.14)	4.83	2	-

Cytogeography

From our Central European dataset the most common ploidy level was tetraploid (72.7%), the second most common one was triploid (21.3%). Other ploidies were very rare (2*x*: 0.8%, 5x: 4.2% and 6x: 0.9%). Tetraploids were ubiquitous, they are distributed across the whole sampling area from lowlands to the highest mountains (195–2860 m a. s. l.; Fig. 6) and are very polymorphic (Fig. 7). Other ploidies were rare and concentrated only in the Alps, except for triploids that were also found in the highest part of the Western Carpathians. All ploidies except tetraploids were found only in higher elevations (1570–2860 m a. s. l.), but without any clear distribution pattern. Diploids were only found extremely rarely in one area (two near sites) of the mountain saddle Staller Sattel (2052 m) on the Austrian-Italian state border by Sankt Jakob in Defereggen, Tirol (Fig. 8).

The sampled *H. selago* populations were mostly ploidy-uniform (72.7%), but approximately a quarter were mixed (27.3%; only populations with more than three samples were counted). The most common uniform populations were of tetraploids (57.6%), uniform populations of the other ploidies were less frequent (2*x*: 0%, 3*x*: 13.1%, 5*x*: 2.0%, 6*x*: 0%). Mixed populations consisted of two or three different ploidies, in various combinations. Diploids were not observed in separated populations and formed a small admixture together among tetraploids or tetraploids and triloids. Similarly, hexaploids did not create separated populations, they were observed in populations together with tetraploids or pentaploids, or with both of them.

Most of the 209 populations were collected on silicate bedrock. Only 15 populations (7.2%) were collected on calcareous bedrock. Most of them were tetraploid, only one population from Slovakia comprised tetraploids and triploids together.

Genome size comparison of live and dried plants

All genome size measurements (Table 1) were obtained from living plants. However, to investigate the potential for the use of this method in further studies, we tested the fluorescence intensity and stability in comparison of live and dried plants. Age of most of dried tissues were similar (on the order of months), therefore this factor was not tested.

In the relative genome size measurements (DAPI stain), there was a significant influence of material (Table 2). Especially the herbarium samples showed a slightly lower relative genome size for both ploidies tested (Table 3).

However, no overlap of individual cytotypes was ever observed (Fig. 9). Both dried tissue types of triploids showed also somewhat wider range of values compared to living tissues. Different trend was observed in the absolute genome size measurements (PI stain). Results were also significantly influenced by type of material (Table 2), however, both types of dried tissues and especially in herbarium samples yielded higher values than living material (Fig. 9). The relative shift was higher in triploids than in tetraploids: +15.2% and + 7.7%, respectively, for silica dried samples, and + 24.3% and + 15.4%, respectively, for herbarium samples.

The type of material influenced significantly CV of the sample peak, which is a measure of the analysis quality (Table 2). In both type of staining silica-gel dried tissues had the highest CV, while herbarium samples had CV similar to live material in DAPI staining and intermediate with PI staining (Fig. 9).

Table 2
Results of ANOVA of influence of type of material (live, silica-dried, herbarium) to relative genome size and sample peak CV (DAPI staining) and genome size and sample peak CV (PI staining). In each analysis a specimen identity was a random factor.

DAPI	**RGS – 3*x***	**RGS – 4*x***	**CV – 3*x***	**CV – 4*x***	**PI**	**GS – 3*x***	**GS – 4*x***	**CV – 3*x***	**CV – 4*x***
F value	10.36	17.59	14.10	10.47	F value	195.50	64.40	87.92	13.96
Df - material	2	2	2	2	Df - material	2	2	2	2
Df - residuals	46	50	46	50	Df - residuals	6	10	6	10
Pr (> F)	0.00019	0.00001	0.00003	0.00016	Pr (> F)	0.00001	0.00002	0.00004	0.00128

Table 3
Relative genome sizes (ratio to the internal standard *Vicia faba*) with DAPI staining and genome size with PI staining for different type of tissue material of *H. selago*.

	ratio to std (DAPI)		**genome size in pg (PI)**	
ploidy	3*x*	4*x*	3*x*	4*x*
live	0.43 (± 0.00)	0.59 (± 0.01)	13.41 (± 0.10)	18.26 (± 0.20)
silica	0.43 (± 0.01)	0.58 (± 0.01)	15.45 (± 0.12)	19.66 (± 0.68)
herbarium	0.42 (± 0.01)	0.58 (± 0.01)	16.67 (± 0.37)	21.08 (± 0.55)

Spore size, spore abortion, and stomata size

Even though *Huperzia* is homosporous, we found that the size and shape of spores in sporangia were not homogeneous. Plants formed the tricolpate spores (regular spores), unseparated tetrads, aborted regular spores, large spherical spores (diplospores), and aborted diplospores - see Fig. 1. Unseparated tetrades were not evaluated, because they were considered as a residuum of unripeness. Only the size of non-aborted spores was measured. In comparison of lengths of regular spores and diplospores, there was a marked difference, diplospores being 1.54× (± 0.11) larger with no overlap in size. There were significant differences between ploidies (one-way ANOVA; regular spores: $F = 19.09$, $df = 4, 32$, $p < 0.001$; diplospores: $F = 21.37$, $df = 4, 28$, $p < 0.001$). In both spore types of diploids had significantly smaller spores than all other ploidies. In polyploids, spore size differed significantly between triploids and pentaploids, while tetraploids and hexaploids were either intermediate or similar to triploids; diplospore in hexaploids were not evaluated due to lack of material (Fig. 10).

The proportion of diplospores (Table 4) was variable (0–19.0%). The lowest proportion was observed in hexaploids (but note that only 2 plants were available) and the highest proportion was in pentaploids. The highest variability between individual plants was observed in pentaploids and diploids.

Table 4
The proportion of diplospores in different ploidies of *H. selago*.

	2*x*	**3*x***	**4*x***	**5*x***	**6*x***
ratio [%]	4.2	2.8	2.8	9.3	0.6
min-max [%]	1.2–14.8	0.6–7.4	0-5.4	2.4–19.0	0–1.0

Spore abortion (SAI), counted from all spores except for unseparated tetrades (i.e., including both regular spores and diplospores), showed difference between ploidy levels (one-way ANOVA, $F = 14.48$, $df = 4, 31$, $p < 0.001$). Tetraploids and hexaploids had lower abortion rate than odd ploidy levels, which also differed from each other (abortion rate in pentaploids being higher than in triploids). Surprisingly, diploids had relatively high abortion rate intermediate between the two odd ploidy levels and significantly higher than both even ploidy levels (Fig. 11)

As a last character, stoma size was measured (Fig. 12). There were significant differences between ploidy levels ($F = 14.85$, $df = 4, 34$, $p < 0.001$). Diploids (42.38 ± 1.60 µm; mean and standard deviation) were different from all other ploidies. Among polyploids, triploids (51.46 ± 2.15 µm) differed from tetraploids (58.92 ± 3.04 µm), while pentaploids and hexaploids were intermediate between them (55.85 ± 6.31 µm and 55.80 ± 3.08 µm, respectively).

DISCUSSION

Distinguishing of cytotypes from genome size and chromosome number

Five cytotypes (2*x*, 3*x*, 4*x*, 5*x*, 6*x*) were detected in Central European *Huperzia selago*. Polyploidization is a common evolutionary process in many lycophytes (Bolin et al. 2018; Xia et al. 2021) with exception of Selaginellaceae, in which polyploidy is very rare (Bainard et al. 2011a). However, ploidy series, such as the one observed in this study, are uncommon in this group of homosporous lycopods. They are usually known in the major spore-bearing land plant group, ferns (e.g., Liu et al. 2019; Hanušová et al. 2019), and in many angiosperms (e.g., Duchoslav et al. 2020) but only few examples were published in lycophytes - in *Lycopodium* (Takamiya and Tanaka 1982), Asian *Huperzia* (Beitel and Wagner 1982) and the heterosporous family Isoëtaceae (Suissa et al. 2022). Discovery of such an extensive polyploidy series in European *Huperzia selago* is indeed not usual.

In this study, *Huperzia* genome size of cytotypes was determined by flow cytometry. Within *Huperzia* genus, there are only several published values so far that belong to North American-Asian diploid *H. lucidula*: 2C = 11.27 pg (Bainard et al. 2011a) and 11.40 pg (Wang et al. 2005). Our lowest record, also for a diploid cytotype (2C = 10.09 pg), corresponds to these values. Similar value was published recently for tetraploid *H. asiatica* 2C = 16.24 pg (Li et al. 2024) but unfortunately with new molecularly established standard, which is not comparable with current data. The largest genome size known to date in lycophytes 2C = 23.94 pg, has been revealed in *Isoëtes lacustris* (Hanson and Leitch 2002). The recently published maxima of 2C values in lycophytes are 27.43 pg and 31.68 pg for *Phlegmariurus squarrosus* and *P. carinatus*, respectively (Wang et al. 2022). In this study the highest values of hexaploidy cytotype of *H. selago*, 2C = 28.39 pg is reaching the maximum lycophyte published genome size values.

Counting of chromosomes (especially in meiotic division) in genus *Huperzia* was considered extremely difficult even by the European leading specialist I. Manton, because of high chromosome number, extreme irregularity of pairing,

chromosome stickiness, long and thin chromatin, formation of micronuclei (Manton 1950). We finally counted mitotic chromosomes in the somatical gemmae roots of three lowest cytotypes. However, because the chromosomes were not ideally condensed and because of high chromosome numbers and various lengths of chromosomes, the counts should be considered approximates.

The expected gametophytic chromosome number in *Huperzia* diploid, which is identical to the base chromosome number of the whole complex, is $n = x = 67$ or 68 (Wagner 1992). This number is very high in comparison with most of other plant chromosome numbers (Rice et al. 2015). The high chromosome number of *H. selago* can be caused by frequent neo- and palaeopolyploidy events, which are supported in lycophytes (e.g. Suissa et al. 2022). Similarly high chromosome counts are common also in ferns, where these events are supported too in ferns (e.g., Schneider et al. 2017). However, genome multiplicities resulting from palaeopolyploidization are not commonly accounted in ploidy multiplicity in spore-bearing plants. Thus the lowest chromosome numbers found in *Huperzia* ($2n = 134$ or 136) are perceived as diploid, based on Wagner (1992). These numbers also correspond with other species, except of *H. selago*, such as *H. lucidula* and *H. serrata* (Beitel and Wagner 1982; Takamiya and Kurita 1983).

The value of the base chromosome number x = 67 results in expected chromosome numbers $2n = 201, 268, 335, 402$ expected for triploids, tetraploids, pentaploids and hexaploids, respectively. The published counts are consistent with this series, although there are minor deviations. For example, chromosome count reported for *H. selago* from Europe is $2n = 264$ (Manton 1950), instead of 268 (counted also in North America by Wagner, 1992). Similarly, our counts for three cytotypes do not exactly match the theoretical values (being $2n \sim$ ca 140, ca 204, ca 262). Whether these deviations stem from dysploidy/aneuploidy or from technical issues (counting in *Huperzia* being extremely difficult, see above, and our results are representing very accurate estimates), remains open question. However, the existence of a continuous ploidy series is indicated by the known chromosome numbers in the genus *Huperzia*: $2n = 134$, 136, 204, 264, 272, 276, and 528 (Manton 1950, Ghatak 1965, Beitel and Wagner 1982, Takamiya and Kurita 1983, Takamiya 1984, Li et al. 2024), where $2n = 134$ is stated also as $2x$ value and $2n = 276$ as tetraploid value. Apart from the values mentioned and discussed here, no other lower numbers were counted, and broader geographical sampling in Northern Europe and other northern regions is possibly needed. However, the reported approach of ploidy discrimination is also in line with discovered chromosome count variability in Asian species (Ghatak 1965, Takamiya and Kurita 1983; Takamiya 1984).

Ploidy distribution patterns

In our cytogeographical study focusing on broader Central Europe, surprisingly five cytotypes in *Huperzia selago* were found. Each of them represents a different ploidy level, while there is virtually no genome size variation within a ploidy level. The geographically most widespread cytotype (tetraploids) also occupies the widest amplitude of environmental conditions. It was almost ubiquitous across the whole sampled area reaching a wide altitudinal gradient ranging 195–2860 m a. s. l. It was dominant on silicate, but also found on calcareous bedrock. On the other hand, the other cytotypes (diploid, triploid, pentaploid and hexaploid) occurred only in the subalpine zone of the Alps and the Tatra Mts (the West Carpathians) at about 1680–2860 m a. s. l. The presence of rare ploidies in subalpine zone can be related to their evolutionary history. For diploids such habitat can be the remainder of their former distribution (Brochmann et al. 2004), and for polyploids it can be free niche which they can colonize thanks their higher tolerance to stress of harsher environment (Rice et al. 2019).

The distribution of *Huperzia* rare cytotypes can be explained from more points of view. Firstly, they can represent relicts survived in refugia similarly as some angiosperm plants (e.g. Schönswetter et al. 2005), that could spread northward after deglaciation (e.g., Abbott et al. 2000). By contrast, they could colonized the Alps from northern regions (Kruk et al. 2015). In the case of the genus *Huperzia* there could be a relation to the reported arctic species *H.*

arctica. This hypothesis would be supported by the parallel distribution observed in the Arcto-alpine lycopod taxa – *Lycopodium clavatum* boreal type (*L. c.* subsp. *monostachyon*), which occurs also in the Alps in the alpine zone (Tribsch and Schönswetter 1999). Finally, the rare cytotypes could be formed uniquely or repeatedly in Central Europe. The hypotheses of independent origin or origins can be supported by existence of mixed populations in higher altitude and no pattern of their distribution. However, to understand properly the right scenario of their distribution and phylogeny, a molecular study is needed.

According the resolved cytotype diversity, it is impossible to detect with certainty the phylogenetic context of the discovered cytotypes and their relationship to northern regions, where several taxa of genus *Huperzia* were reported (*H. acicularis, H. arctica, H. conntinetalis, H. europea, and H. selago*). The main problem is the identification of *Huperzia* taxa in general. Its simple frond morphology without distinct identification characters makes it almost impossible to morphologically delimit individual taxa. The situation is probably strongly affected by very high morphological plasticity of plants growing in different environmental conditions (Löve and Löve 1965). In another point of view, even ploidy level or chromosome counts do not help to determine the taxa. They have not been clearly given for any of these taxa mentioned above, except *H. selago*, which was the only one to have its chromosomes validly counted, but ploidy has not been reported. In contrast to this condition, in the several European floras (Valentine and Moore 1993; Blockeel 2006) a lower chromosome count was assumed for *H. arctica* (2n = 90) and a higher chromosome count for *H. selago* (2n = 264), but it is not supported by primary data. Although the cytotype variation we found may be related to the theoretically with different numbers of chromosomes, sampling will need to be extended to northern regions (Vejvodová and Ekrt under study) to determine the actual distribution, morphology and relationships of the various types and their relationship to *Huperzia* taxa stated in Europe. For now, we consider the polyploid series discovered in the mountains of Central Europe under *H. selago* sensu lato.

Measurability of dried tissues

To further study the genus *Huperzia*, including transporting samples from more distant areas, we tested the applicability of genome size estimation by flow cytometry from dried tissues. Based on our results, ploidy determination from silica-dried and herbarium samples is reliable using DAPI-stain, although lower quality of the analyses was observed, namely a reduction in signal intensity and a higher coefficient of variation. This is comparable to previous findings in flowering plants (Schönswetter et al. 2007), even in ferns (Wolf et al. 2015).

In analysis of herbarium even silica-dried tissues higher CV was a significant factor causing increasing in genome size (with PI-stain). An increased CV has been noted also in other study (Tomaszewska et al. 2021) and some authors do not even recommend using dried material for such precise whole genome measurements (Doležel and Bartoš 2005). Caution is in order here, because an increase in genome size using dried samples in our dataset was observed - similarly as in Bainard et al. (2011b). In other hand, different situation was noted were achieved in lycophytes, in the genus *Selaginella*, where both herbarium and silica dried tissues aged 14.4 years were successfully used to measure the absolute genome size (Little et al. 2007). However, there was in our data a large difference in the type of dried material, while herbarium specimens showed greater shifts in genome size than silica-dried tissues. Rarely even an overlap of ploidies was found (in the case of the genome size of herbarium tetraploid and living triploid). Therefore, genome size measurement obtained from dried *Huperzia* tissue should be used carefully, without direct comparison of live and dried plant values.

Developed and aborted spores in lycophytes

In ferns, well established species (usually having even ploidies) have well developed spores, while hybrids are generally almost completely aborted, which is a result of irregular meiosis after hybridization (Wagner et al. 1986).

Similarly, in angiosperms the pollen (non)viability often reflects meiotic problems in hybrids or odd ploidy levels (e.g., Zhang et al. 2006). We therefore tested if spore viability correlates with ploidy level in the *Huperzia* cytotypes.

The assessment of the well-developed and aborted lycophyte spores is complicated by thick spore wall (e.g., Ramos Giacosa et al. 2016). Therefore, there is almost no rule for recognizing abortive and viable spores. Examples of description of abortive spores were published only a few times for megaspores of *Isoëtes* (Wagner et al. 1986) and as histochemical difference of abortive and fertile spores in *Lycopodium* and *Diphasiastrum* (Alexander 1969). Abortive megaspores were detected in hybrids and odd ploidies in *Isoëtes* (Suissa et al. 2022), where an odd ploidies individually produced 99% of aborted megaspores (Taylor in Wagner et al. 1986), and in *Huperzia* (Beitel and Mickel 1992), where triploids aborted 95% of their spores (Takamiya 1984).

In general, our data indicated a trend similar to the assumed one, that odd ploidies have high spore abortion potential. Even ploidies (4x, 6x), except diploids had 31.8% and 11.8% respectively of spore abortion, while odd ploidies (3*x*, 5*x*) had 55.0% and 75.4%, respectively, of spore abortion. However, spore abortion of odd ploidies was not as high as observed in *Isoëtes* and *Huperzia*. Additionaly, pure ploidy distinguishing was also limited because diploids surprisingly had an extremely high spore abortion about 70.2%. This is comparable to odd ploidies. It is difficult to explain with our current knowledge and it may be probably related to undersampling of this ploidy (only 10 plants from one area) connected with extremely rare occurrence and possible genetic drift within small population of diploids (Spielman et al. 2004). The higher proportion of aborted spores could also be caused by abiotic stress (Arosa et al. 2009) or long-term isolation of a small population and possible inbreeding (Simiqueli et al. 2018). Both hypotheses are supported by the fact that diploids have only been found in Central Europe at one small site in the central Alps. We could speculate that the diploids survived in very limited population number whereas tetraploids were more successful and became widespread after last glacial maximum, as in some examples of ferns (Vogel et al. 1999).

Distinguishing of cytotypes via spores and stomata

In ferns, the spore and stoma size are usually correlated with ploidy level (Ekrt et al. 2021). In angiosperms a similar trend is known with pollen and stomata (e.g., McGoey et al. 2014). These correlations are assumed also in lycophytes (Bolin et al. 2018) but there is still lack of data. Only a few studies focusing on stomata and spore size were published in genus *Isoëtes.* In general, ploidy did not correlate with stoma and megaspore size. On the contrary, there appeared a positive correlation of ploidy and microspore size (Troìa 2001). In contrast to these results, our data confirm significant differences in stoma size only among diploids, triploids and tetraploids and in spore size among diploids and the other ploidies. Surprisingly, samples with higher ploidy levels show no difference in these microcharacters.

Besides regular (haploid) spores, other bigger spherical spores were also found in our study. On average, their size is about 51–60% larger than regular spore size. Such spore formations were observed in *Huperzia* in the past only very sporadically and, in this case, they were considered to be spore mother cells (Beitel and Mickel 1992), the diploid (2*n*) precursors to haploid spores. However, the bigger spherical spores were noted in lycophytes even in genera *Diphasiastrum* (Schnittler et al. 2019) and *Lycopodium* (Takamiya and Tanaka 1982), but their cytological character is still not clear. Schnittler et al. (2019) and Wagner (1986) discussed a possibility of their unreduced ploidy level and their role in life cycle. Such unreduced spores could represent spore mother cells or diplospores. In ferns, diplospory is the predominant process of spore formation in fern apomicts (Manton 1950; Grusz 2016), and, to a lesser extent, their hybrids with sexual species (apo-sex hybrids; Hornych et al. 2022). Diplospores also occur in regular sexually reproducing species (Ekrt et al. 2021), but the proportion of unreduced spores is likely very low (Nakato and Masuyama 2021). If the role of bigger spherical spores in lycophytes should be evaluated, special attention must be paied to many aspects of lycophyte reproduction biology such as sporogenesis, ploidy level of spores, their viability and their gametophytes biology.

Declarations

Competing Interests

None.

Declarations

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Figures

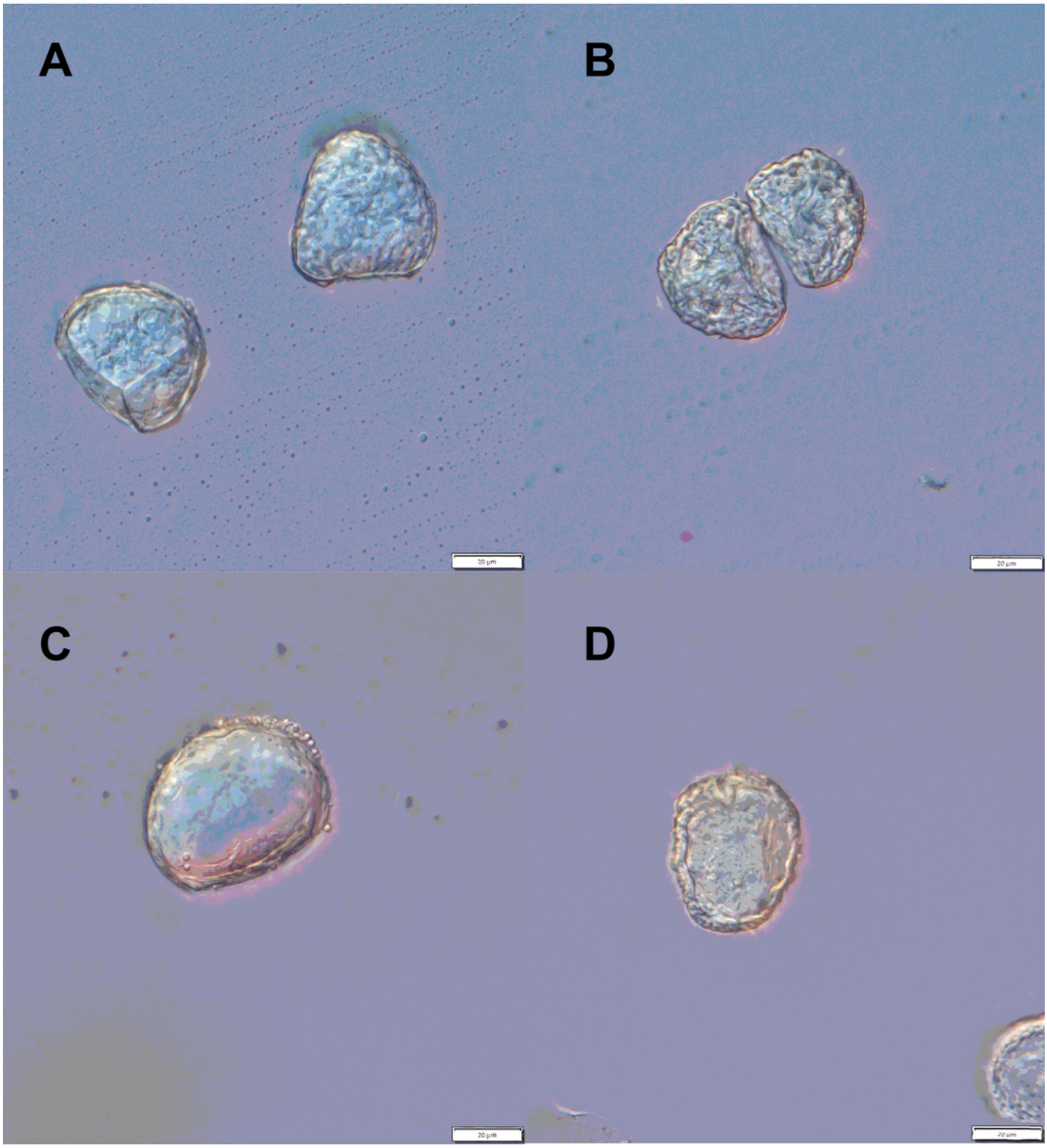

Figure 1

Types of spores under evaluation (a) viable regular spores, (b) aborted regular spores, (c) viable diplospores, (d) aborted diplospores in *Huperzia selago*using acetocarmine stain. Scale bar = 20 µm.

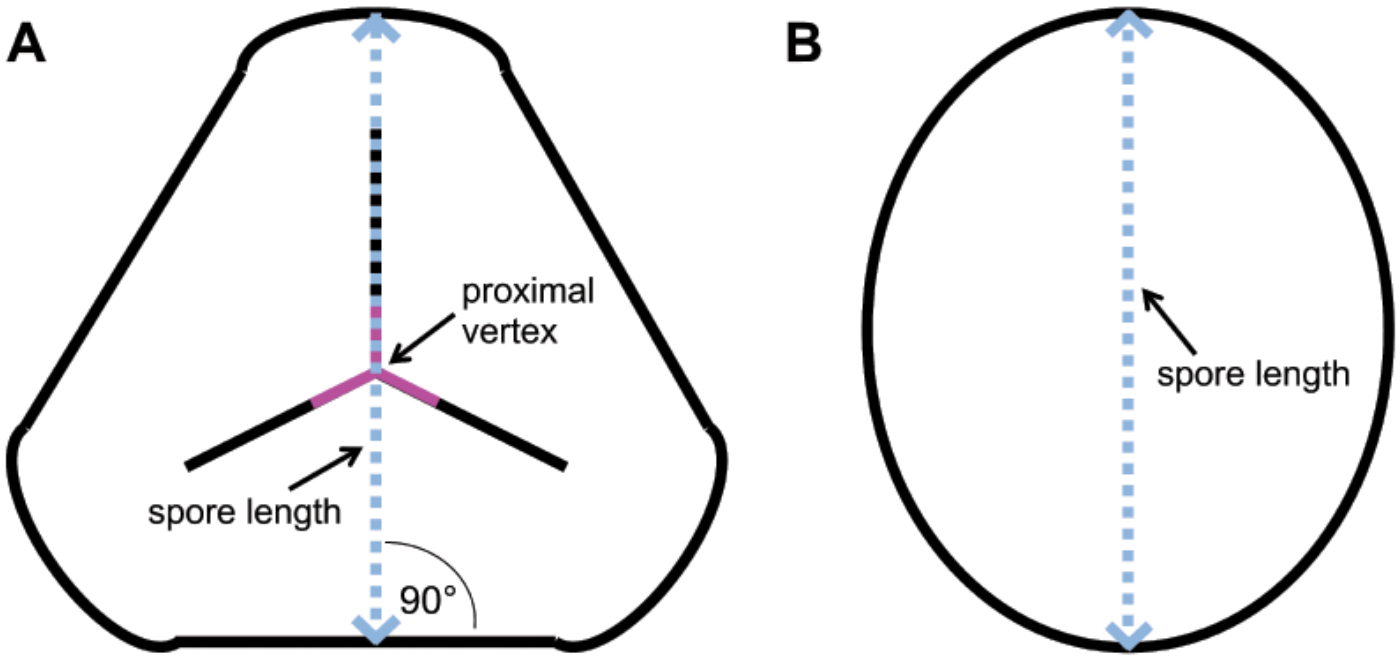

Figure 2

Schema of spore length measurement: (a) The pink lines indicate the center of the trilete scar, visible on the proximal side of the spore. Measured spore length represent blue dotted line. (b) Measurement of diplospore length.

Figure 3

Illustrative pictures of *Huperzia selago* cytotypes, (a) diploid, locality ID 159 (Rasen-Antholz, the Alps, Italy, (b) triploid, locality ID 30 (Hohentauern, Niedere Tauern Mts, Austria), (c) tetraploid, locality ID 160 (Anholtz-Mittertall, the Alps, Austria), (d) pentaploid, locality ID 117 (Matrei in Osttirol, Hohe Tauern Mts, Austria), (e) hexaploid, locality ID 84 (Andermatt, Glarner Alpen, Switzerland). All photos L. Ekrt. For locality ID see Supplement SI1.

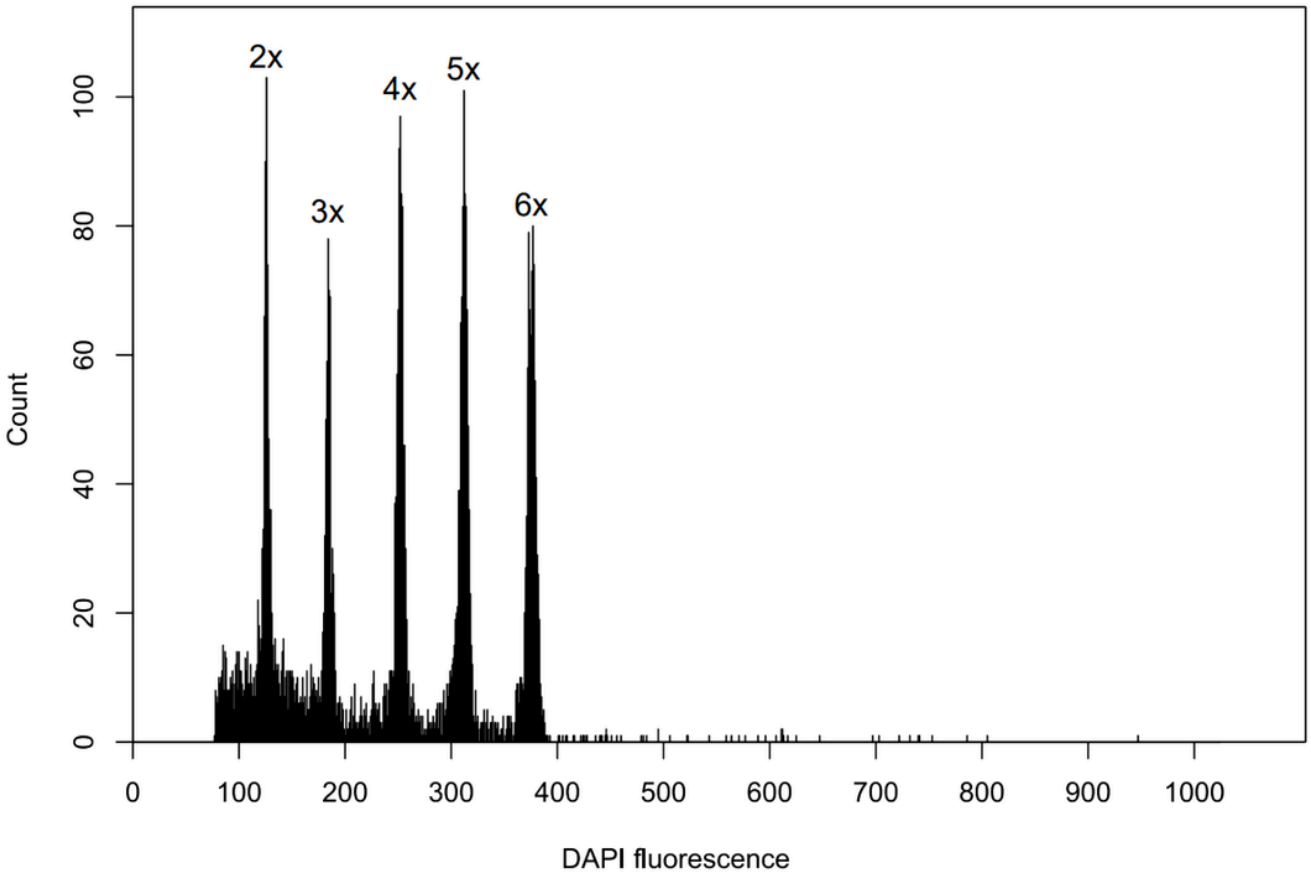

Figure 4

Flow cytometric histogram showing a simultaneous analysis of 5 detected ploidy levels of *Huperzia selago* measured from the fresh plants coming from the Central Europe

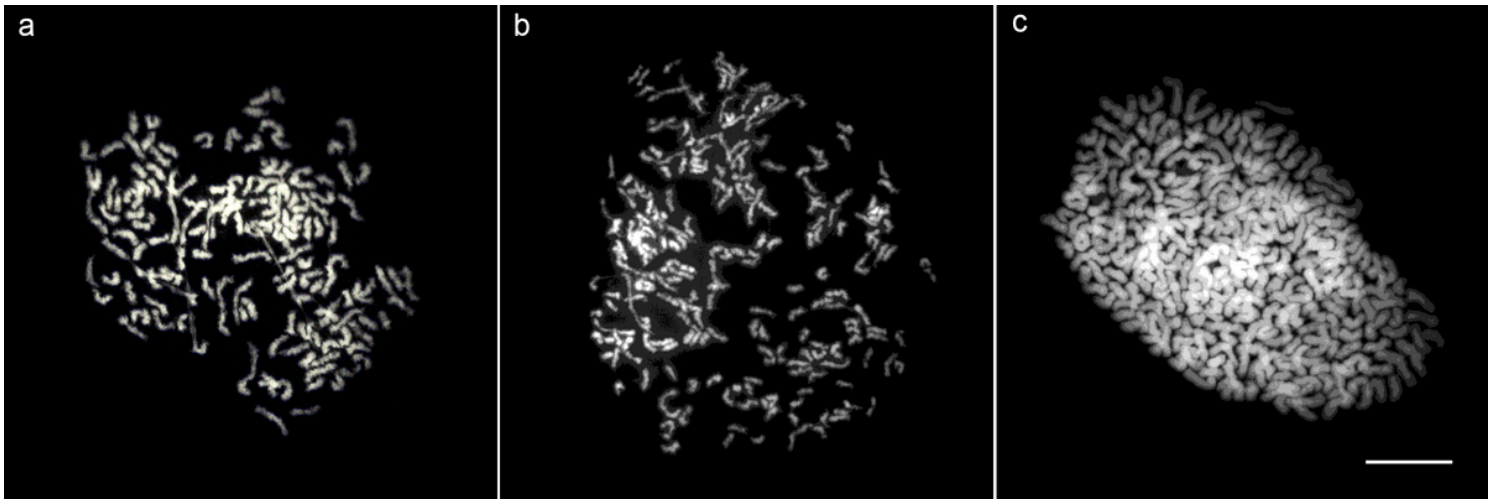

Figure 5

Mitotic chromosome spreads of *H. selago* under fluorescent microscope: (a) diploid, 2n ≈ 137 chromosomes, locality ID 108, plant 2 (Rasen-Antholz, the Alps, Italy); (b) triploid, 2n ≈ 204 chromosomes, locality ID 102 (Tatranská Lomnica, Vysoké Tatry Mts, Slovakia); (c) tetraploid 2n ≈ 262 chromosomes, locality ID 45 (Uricani, Retezat Mts, Romania); scale bar = 10 μm. For locality ID see Supplement SI1.

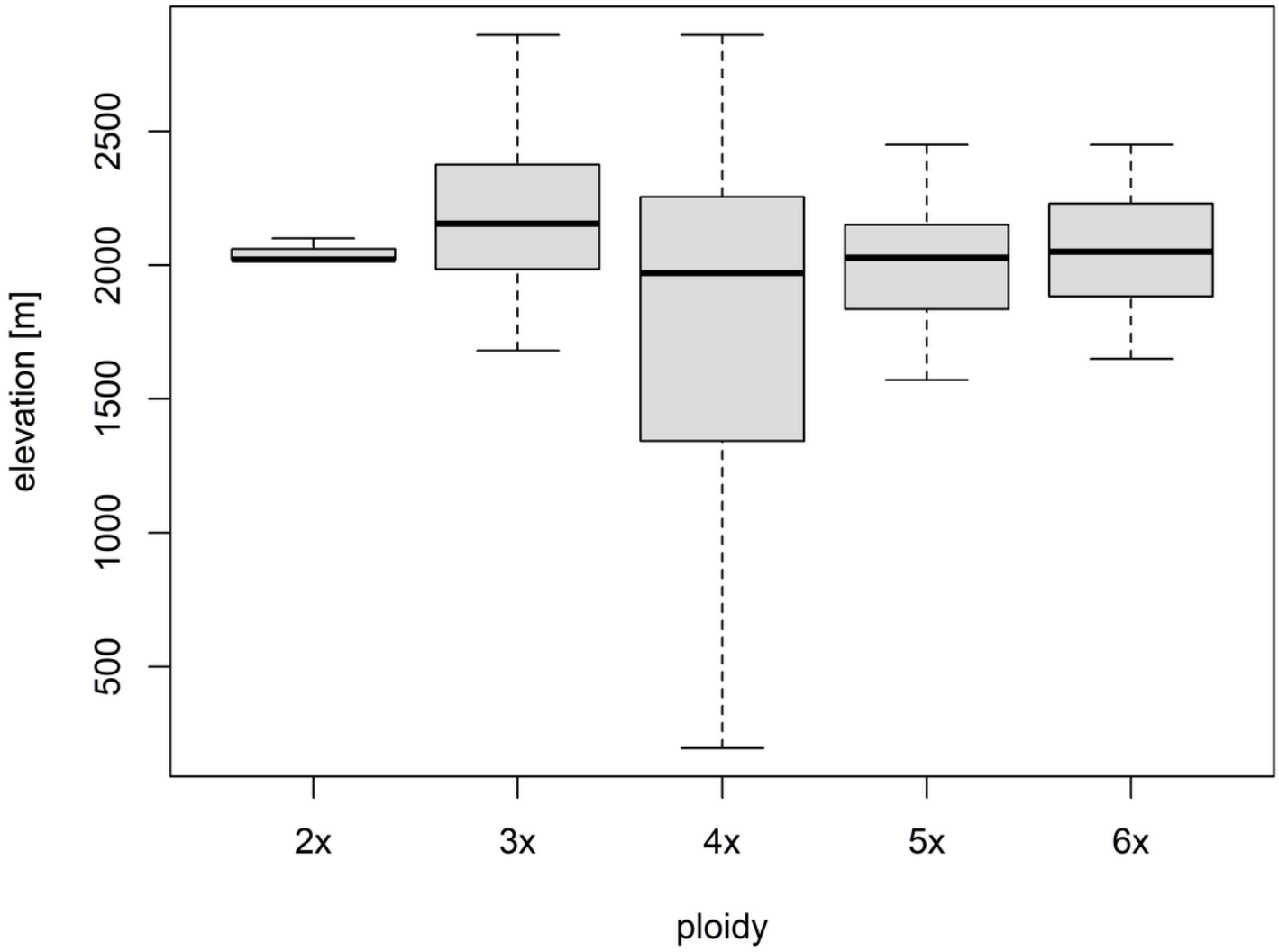

Figure 6

Distribution of *Huperzia selago* ploidies on altitude gradient.

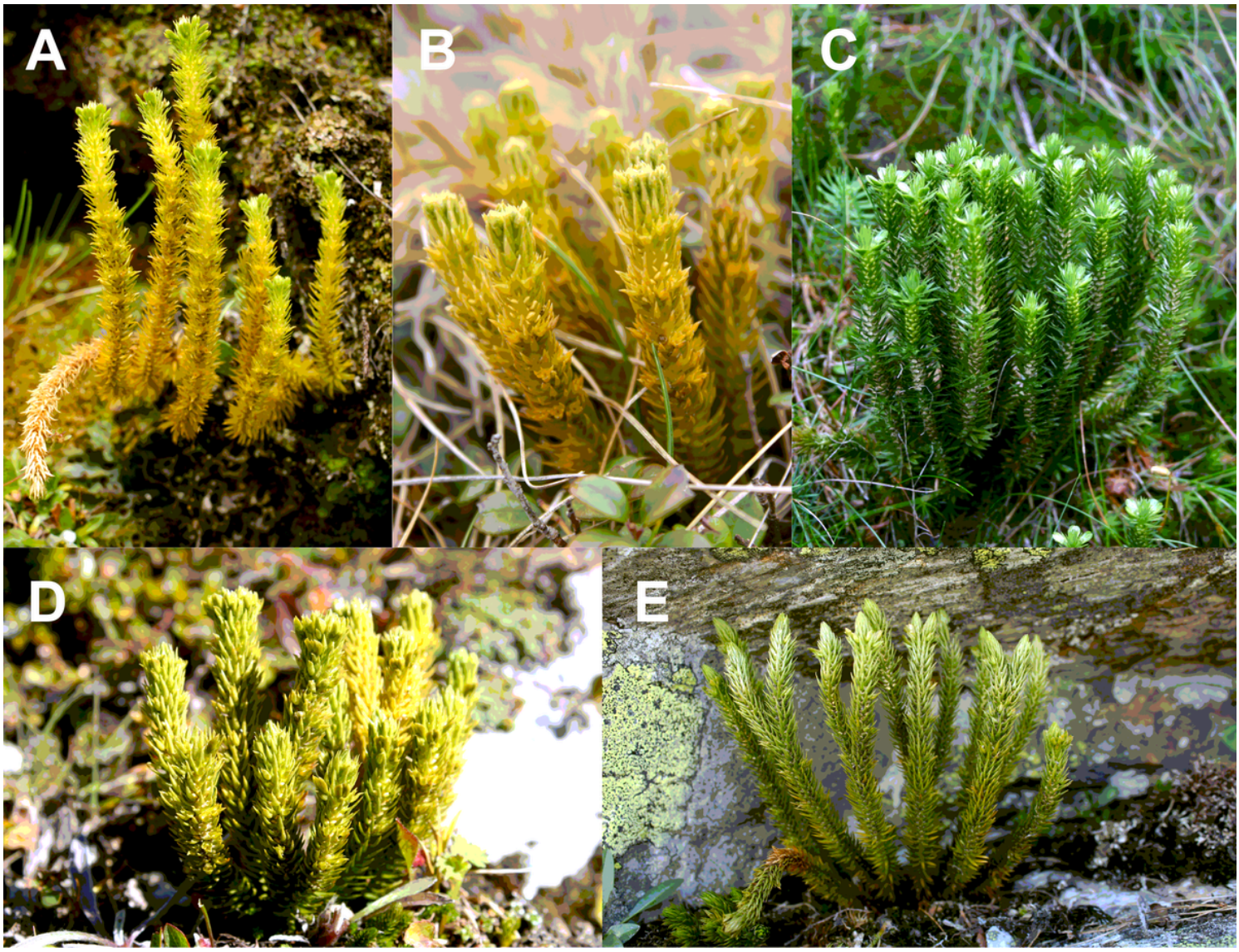

Figure 7

Illustrative pictures of morphological variation of *Huperzia selago* tetraploids (a) locality ID 160 (Anholtz-Mittertall, the Alps, Austria), (b) locality ID 111 (Sölden, Ötztaler Alpen, Austria), (c) locality ID 157 (Hamry, Šumava Mts, Czech Republic), (d) locality ID 89 (Termignon, Massif du Mt Cenis, the Alps, France). All photos L. Ekrt. For locality ID see Supplement SI1.

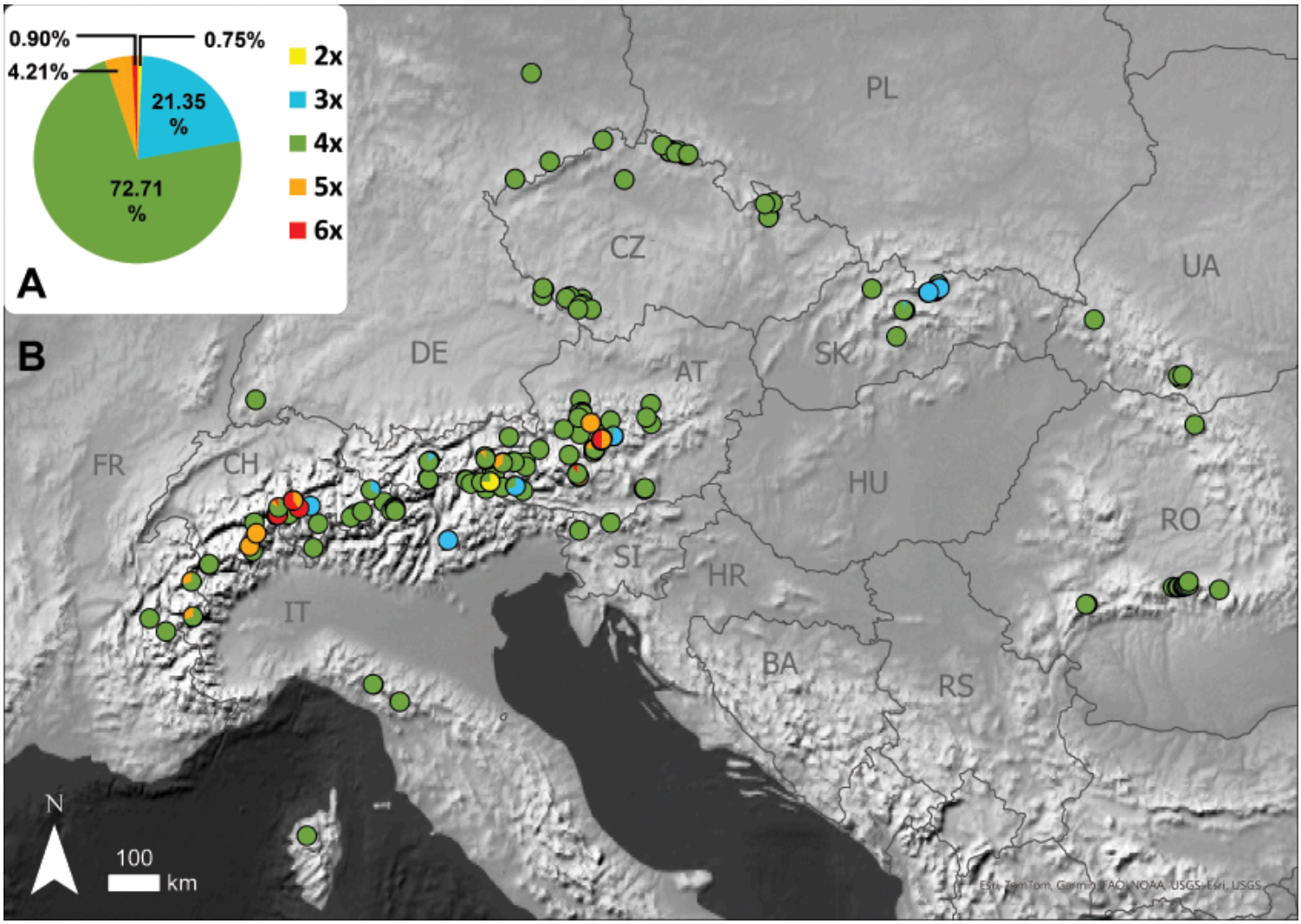

Figure 8

Cytotype proportion in the study area (a) and spatial distribution of cytotypes (b) of *H. selago* in Central Europe. Mixed-ploidy populations are depicted as pie charts showing the local frequency of DNA ploidy levels (RGS). Entirely tetraploid populations (green) are situated in the back of other cytotypes for improved clarity.

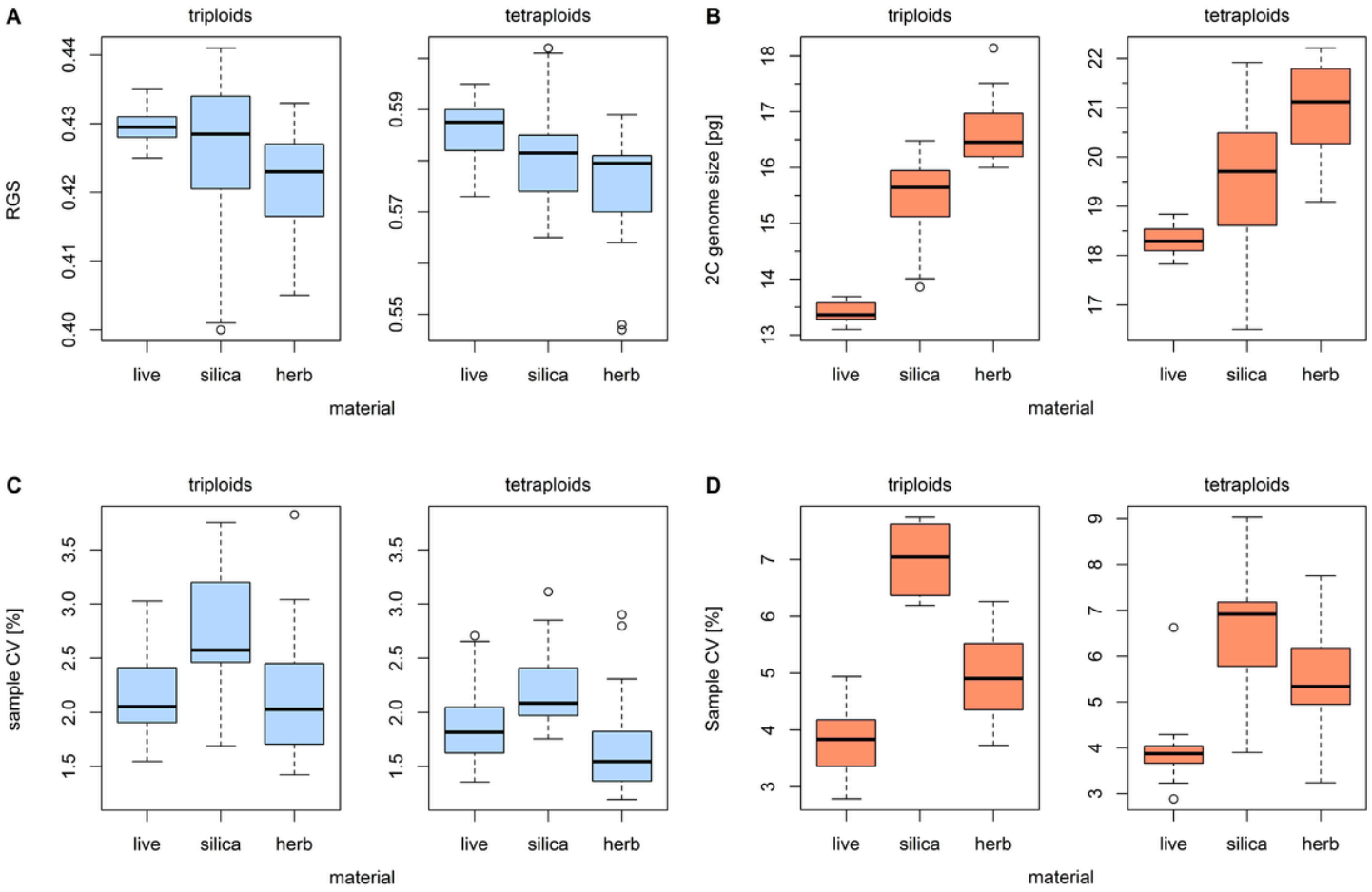

Figure 9

Comparison of flow cytometry results with different type of material analyzed (live = fresh living tissue, silica = silica-dried tissue, herb = air dried tissue from herbarium vouchers) in triploid and tetraploid cytotype of *H. selago*: (a) relative genome size (DAPI staining), (b) genome size (PI staining), (c) sample peak CV (DAPI staining), (d) sample peak CV (PI staining). Blue = results of relative genome size (RGS) measurements with DAPI staining, Red = results of genome size measurements with whole genome PI staining.

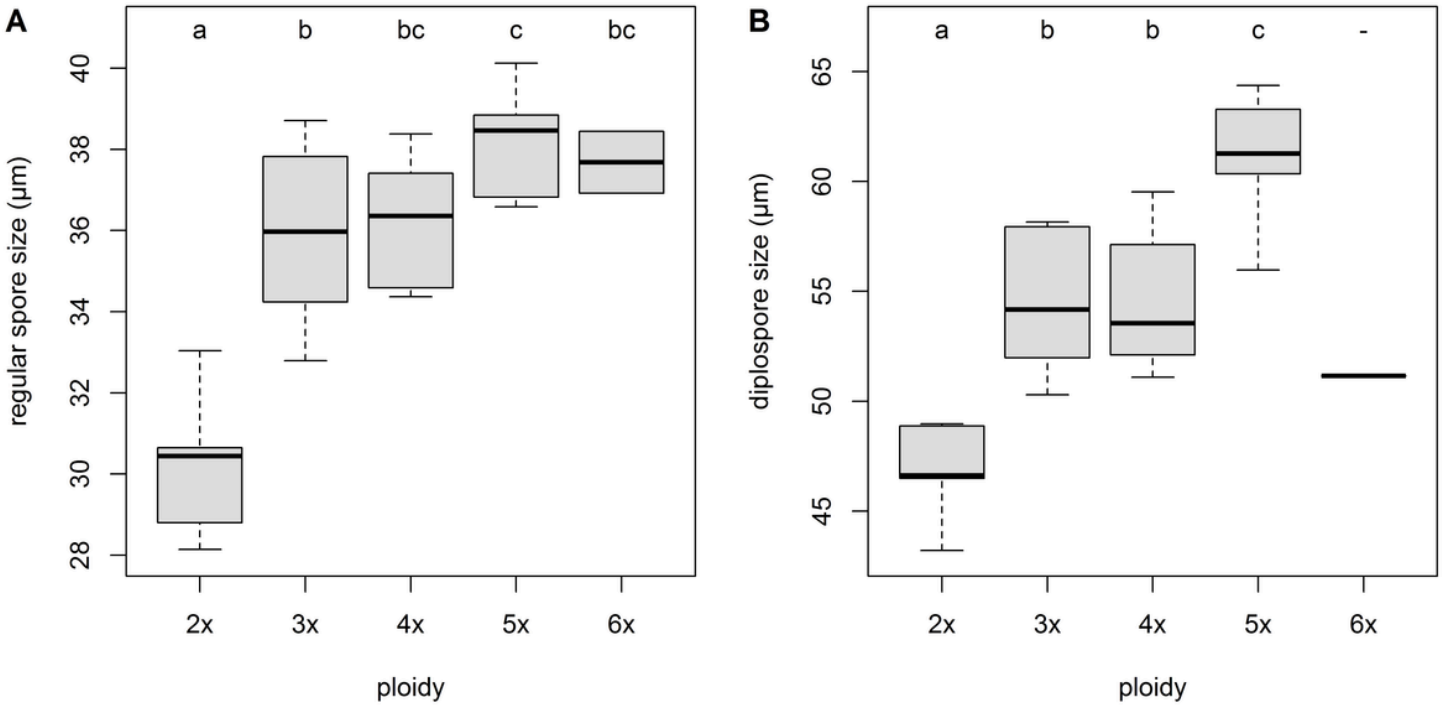

Figure 10

Spore size in different ploidies of *H. selago,* (a) regular spores, (b) diplospores. Letters above boxes denote differences in Tukey HSD test at p = 0.05.

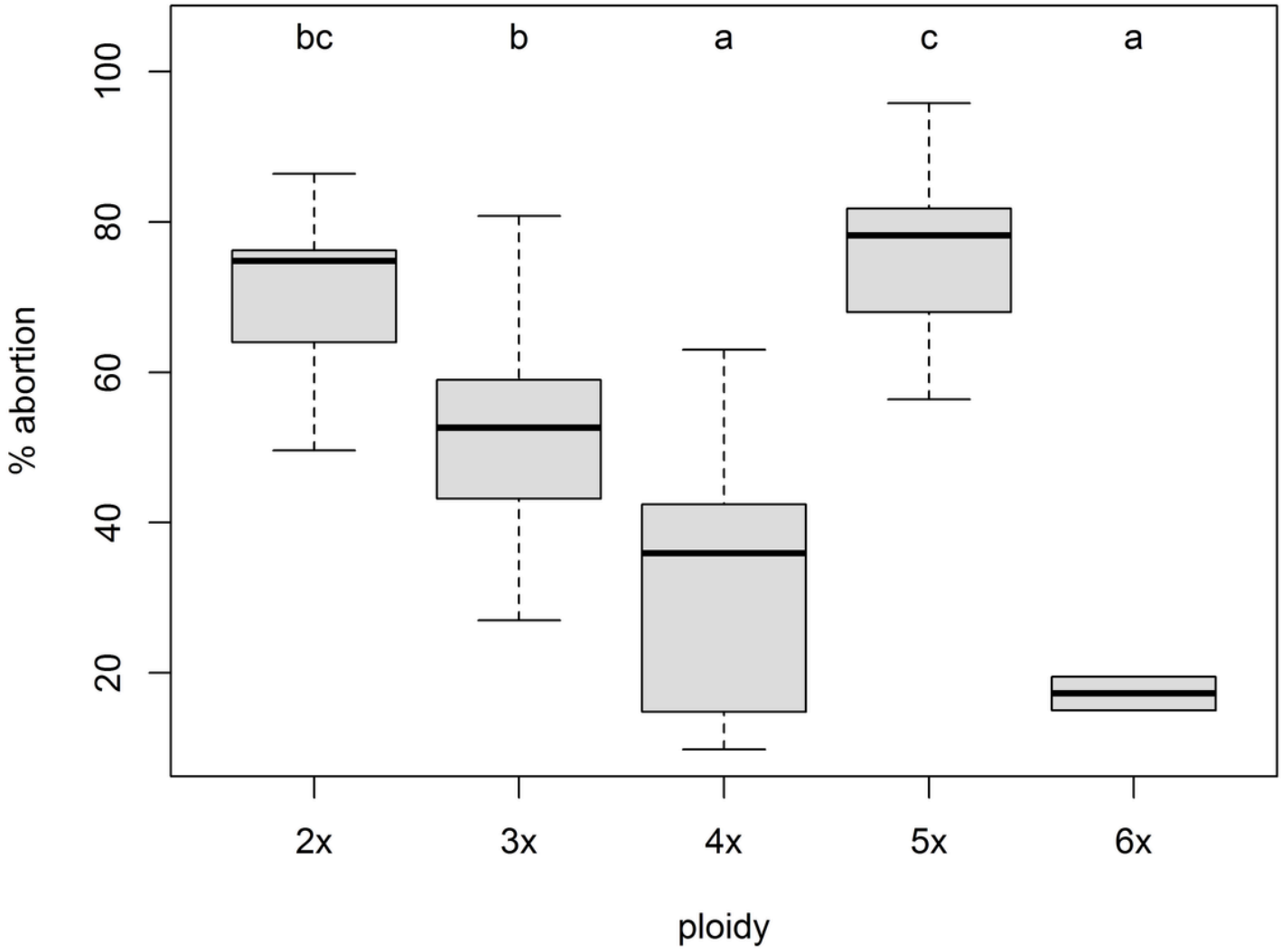

Figure 11

Proportion of aborted spores (SAI) detected in *H. selago* ploidies. Letters above boxes denote differences in Tukey HSD test at p = 0.05.

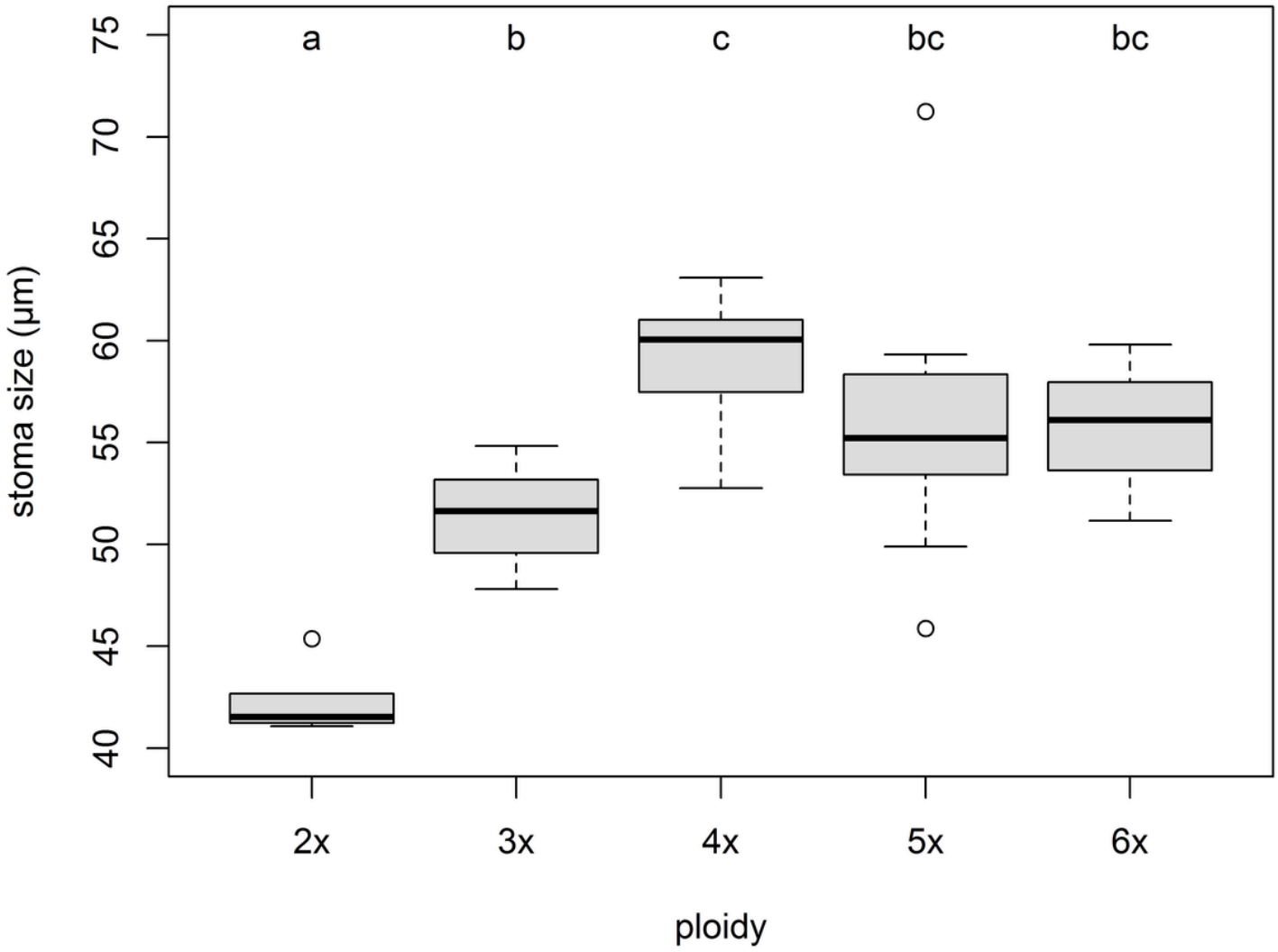

Figure 12

Stoma size of *H. selago* ploidies. Significance of their differences was tested by Tukey test.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigS1.tif
- FigS2.tif
- Supplementarydata.xlsx