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SHORT COMMUNICATION

Variability of stem morphology in *Lycopodium clavatum* (Lycopodiaceae) is not related to ploidy level

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Background and aims – Based on pedicel length, three morphological forms of *Lycopodium clavatum* L. are recognized. It has been previously reported that these are correlated with the ploidy level. The present investigation compares for the first time in Poland the diversity in pedicel length with the corresponding nuclear DNA amount and the ploidy level of *L. clavatum*. We aimed to find correlation between the morphotypes and the ploidy level in the Polish population of *L. clavatum*.
Methods – Samples of shoots were taken from two clumps of *L. clavatum*. The ploidy level and 2C DNA content analysis was combined with pedicel length measurements. The ploidy level and genome size estimation were performed using flow cytometry.
Key results – We found the occurrence of two morphological types of generative stems. The morphotype A produced distinct long pedicels (10.8–43.3 mm). The morphotype B had visible, but very short pedicels (4.5–9.3 mm). We tested the already existing hypothesis, according to which the A and B morphotypes represent respectively diploid and triploid ploidy level. Surprisingly, we did not find such a correlation. Regardless of the morphotype, all the studied plants were diploid, with 5.04–5.13 pg/2C nuclear DNA content.
Conclusions – The occurrence of stems of different morphology in *L. clavatum* was not linked to the ploidy level. It is not excluded that the morphotypes may be genetically determined, but in the studied material, the ploidy level is certainly not at the origin of their differentiation.

Key words – Club moss, DNA content, flow cytometry, morphotype, sporophyte, strobilus.

INTRODUCTION

Lycopodium clavatum L. (common clubmoss) is an evergreen plant with dichotomously dividing stems and small, alternately arranged entire leaves. It is broadly distributed throughout the world, occurring in various habitats. The species shows a wide plasticity of morphological characters, introducing much confusion in the taxonomical assignment. Taxonomic problems are even more serious because of the wide geographical distribution, intrapopulation variability, the existence of morphologically intermediate forms, as well as the clonal growth habit (Takamiya & Tanaka 1982). Although many morphological traits have been considered in previous investigations, the length of pedicels revealed to be the most significant taxonomic character in *L. clavatum* (Takamiya & Tanaka 1982). For example, according to Nakai (1925, cit. after Takamiya & Tanaka 1982) pedicel length is suitable for discrimination between *Lycopodium clavatum* var. *nipponicum* and var. *robustius* in Japanese populations. The former variety has obvious pedicels while the latter has pedicels extremely short or lacks them completely. Interestingly, according to Takamiya & Tanaka (1982), a morphological and cytological intrapopulation discontinuity exists in the Japanese common clubmoss. Based on the pedicel length, the authors recognized three distinct morphological forms and correlated them with the ploidy level. While the diploids (2n = 2x = 68) have the morphotype with distinct, long pedicels (10–30 mm), the tetraploid cytotype (2n = 136) possesses the morphotype with pedicels extremely short (0.1–0.3 mm) or absent. The intermediate triploid cytotype

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(2n = 102) belongs to the morphotype which is characterized by obvious, although very short pedicels (1–10 mm). The most frequently estimated number of chromosomes is 68 for diploid forms, 102 for triploid and 136 for tetraploid cytotypes (Manton 1950, Mehra & Verma 1957, Sorsa 1961, Sorsa 1963, Löve & Löve 1966 and literature cited there, Takamiya & Tanaka 1982, Takamiya 1989 cit. after Takamiya 1992, Takamiya 1992, Wagner 1992 Tutin et al. 2010). Since polyploidy in pteridophytes occurs frequently (Bennett & Leitch 2001, Wood et al. 2009), its relation to speciation has been widely discussed (cf. Lovis 1978, Aagaard et al. 2009). Nuclear DNA content is an important biodiversity character with fundamental biological significance. Genome size has numerous practical applications at many different levels (Bennett et al. 2000, Obermayer et al. 2002). However, in the common clubmoss, studies on the morphological variations correlated with the genome size are rare (Takamiya & Tanaka 1982, Hanušová et al. 2014). Notably, Takamiya & Tanaka (1982) suggested that diploids are expected to represent *Lycopodium clavatum* var. *nipponicum*, the tetraploids – var. *robustius*, and the triploids – an intermediate variety. However, these findings were not supported by any further investigation on *L. clavatum*. As to the European populations of *L. clavatum*, there is no precise information on morphotypes and pedicel length, and neither morphological diversity nor the ploidy level have been previously analysed.

The goal of this preliminary investigation was to assess for the first time in Poland, the pedicel length diversity along with simultaneous verification of the nuclear DNA amount and the ploidy level within two clumps of the common clubmoss (*L. clavatum*). Particularly, we aimed to verify the existence of both the morphological discontinuity and the claimed correlation between A (with distinct, long pedicels 10–30 mm) and B (with very short pedicels 1–10 mm) morphotypes, and the ploidy level of the analysed individuals. If such a correlation exists, it can be potentially useful for taxonomic assignment of the species. Our methodology included visual determination of morphotypes and their morphometric analysis across two localities, as well as the nuclear DNA content estimation using the flow cytometry.

MATERIALS AND METHODS

Plant material and research strategy

The studied *Lycopodium clavatum* sporophytes grew at two localities in the Southern Poland in the forest division of Olkusz – locality number 1: N50 20.073 E19 28.279, locality number 2: N50 19.575 E19 28.183. The specimens of *L. clavatum* from two analysed localities are deposited in the Herbarium of the University of Silesia (KTU): sample from locality number 1 - KTU 148563, sample from locality number 2 - KTU 148564. The inhabited forests were coniferous, representing *Dicrano-Pinion* classes, which are typical habitats of *L. clavatum* (Matuszkiewicz 2001).

The ploidy level and 2C DNA content combined with the pedicel length measurements were performed on the erected stems growing from the same horizontal stem. The generative stems were collected from the scattered spots throughout each locality. The distinction of morphotypes was carried out on the basis of pedicel length measurements, which compromised the average pedicel length ranges given by Takamiya & Tanaka (1982): 10.1–30 mm for the morphotype A, 1.1–10 mm for the morphotype B, and 0–1 mm for the morphotype C. The lengths of the pedicels from the stem with one peduncle were averaged and the obtained mean value served as the basic measurement data to assess the morphoptype of a given stem. Totally 13 horizontal stems and 31 generative stems were analysed.

Chromosome number determination

Since the locality 1 showed the presence of type A stems (see Results), it was initially chosen as a diploid reference standard for flow cytometry estimation of the ploidy levels (see below). Thirty roots were excised from four horizontal stems, which grew widely separated from one another. Material treatment and fluorescent chromosome staining with DAPI (4'6-diamidino-2-phenylindole) were as described previously (Golczyk 2011). Totally, chromosome number in 55 best metaphases was estimated under 100x immersion objective.

Flow cytometry analysis

The ploidy level and genome size estimation were performed using flow cytometry. Plant material consisted of the fresh and young leaves of *L. clavatum* from vegetative stems, as described above. Ploidy level was estimated based on the comparison of the position of the G_0/G_1 peak of the target sample on the histogram with the diploid reference standard of *L. clavatum*. The nuclear DNA content of *L. clavatum* was estimated using *Vicia villosa* 'Minikowska' leaves as an internal standard (3.32 pg/2C; Dzialuk et al. 2007). Plant material was prepared according to Zenkteler & Jędrzejczyk (2012). Nucleus isolation buffer was supplemented with DAPI (2 μg/mL) for ploidy level, or propidium iodide (50 μg/mL) and ribonuclease A (50 μg/mL) for genome size estimation. Ploidy level was analysed using a Partec CCA flow cytometer, equipped with mercury UV lamp. Nuclear DNA content was measured using a Partec CyFlow SL Green flow cytometer (Münster, Germany). The 2C DNA content for all the analysed samples of *L. clavatum* was calculated using the linear relationship between the 2C peak positions of the target species and *V. villosa* on the histogram of fluorescence intensities. The mean coefficients of variation of the 2C nuclei content were estimated for all samples of *L. clavatum.* Mean values and standard deviations (SD) of the genome size were estimated for all the samples.

RESULTS AND DISCUSSION

The length of pedicels from the locality 2 was similar to the pedicel length of triploid morphotypes previously reported by Takamiya & Tanaka (1982), without reaching the ranges' limits. The length of pedicels from locality 1 was within the range of 10.8–43.3 mm. The maximum pedicel length was higher than the Takamiya & Tanaka's diploid morphotype: 10–30 mm. The mean value for the morphotype A collected from locality 1 was nearly three times higher than the mean for the morphotype B originated from locality 2 (table 1).

Table 1 – **Length of pedicels, morphotype and nuclear DNA content (pg/2C) of *Lycopodium clavatum*.**
No, locality number; M.S., material sampling: number of horizontal stems / number of generative stems / number of pedicels; M, morphotype.

No.	M.S.	Length of pedicels (mm)		M	pg/2C ± S.D.
		average ± S.D.	range		
1	3/20/40	19. 58 ± 8.59	10.8–43.3	A	5.13 ± 0.01
2	10/11/28	7.05 ± 1.63	4.5–9.3	B	5.04 ± 0.03

All the stems from locality 1 represented morphotype A and all the stems from locality 2 represented morphotype B, being uniform within the clumps. In clubmoss, a non-uniform population structure seems to be a common occurrence (Takamiya & Tanaka 1982).

The somatic chromosome number (2n) for locality 1 was invariably set at 68 chromosomes (fig. 1), therefore confirming its diploid status and usefulness as a reference standard for ploidy level estimation by flow cytometry. Our investigation revealed that regardless of the morphotype, all the thirteen analysed clubmoss samples (from thirteen horizontal stems from localities 1 and 2) had the same ploidy level, and were diploid (fig. 2A) The histograms of the genome size (fig 2B) showed two distinct G_0/G_1 peaks with mean coefficient of variation (CV) values for the *L. clavatum* peak ranged from 3.07% (locality 1) to 3.82% (locality 2). The 2C DNA content ranged from 5.04 pg (locality 2) to 5.13 pg (locality 1), and was lower than 5.71 pg/2C, previously estimated using FCM/PI (100 µg/mL), *Pisumm sativum* as an internal standard, and LB01 isolation buffer supplemented with 1% PVP40 (Bainard et al. 2011). According to Loureiro et al. (2005) some differences in the measurements of DNA content can occur among laboratories and may be caused by using different estimation methods, nuclei isolation buffers, DNA staining fluorochromes or internal standards. The presented results allow us to state that the occurrence of stems of different morphology is not strictly related to the ploidy level. We do not exclude the option that the *L. clavatum* morphotypes might be genetically determined, but in the studied clumps of clubmoss the ploidy level was certainly not at the origin of this variability. The ploidy level often can be linked with the diversity of micromorphological traits in plants (Gola & Szczęśniak 2012). For example, spore size and stomatal cell size were used as a suitable marker for inferring changes in ploidy level among closely related species (Moran 1982, Beck et al. 2010, Gabriel y Galán et al. 2011, Dyer et al. 2013). Nevertheless, our study revealed that such correlation certainly does not exists between pedicel length and nuclear DNA content in *L. clavatum*.

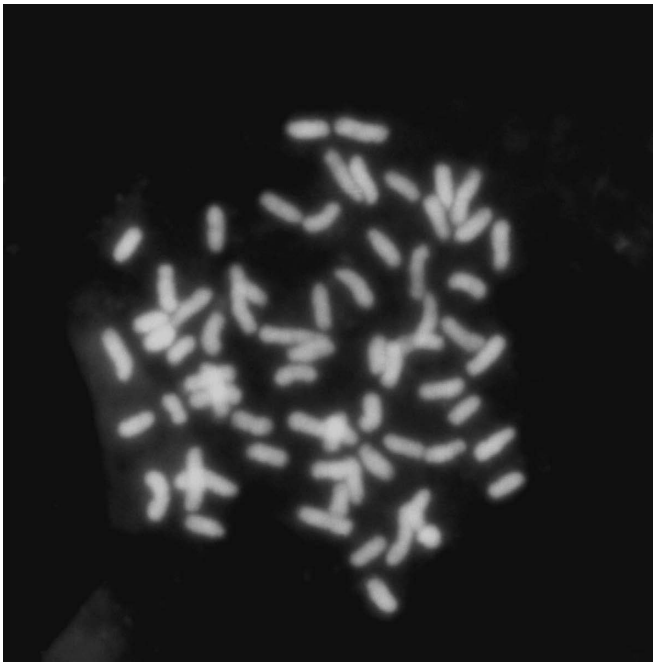

Figure 1 – A root tip metaphase plate of a *Lycopodium clavatum* clone with 2n = 2x = 68 chromosomes from the type A; locality 1, constituting the diploid reference standard for the flow cytometry analysis.

Environmental conditions of a habitat, including the amount of light available for plant photosynthetic apparatus can introduce significant alterations into the morphology of plant stems, also in Lycopods. For example, it is impossible to identify the *Diphasiastrum alpinum* species correctly when it grows in a very strong shadow (Pacyna 2006, Hanušová et al. 2014). However, the diversity of strobilar organ morphology coupled with the apparent uniformity of the ploidy level, cannot be explained by light conditions. None of the two studied localities of *L. clavatum* were placed under extremely dense canopy of trees or shrubs that could significantly alter the amount of light.

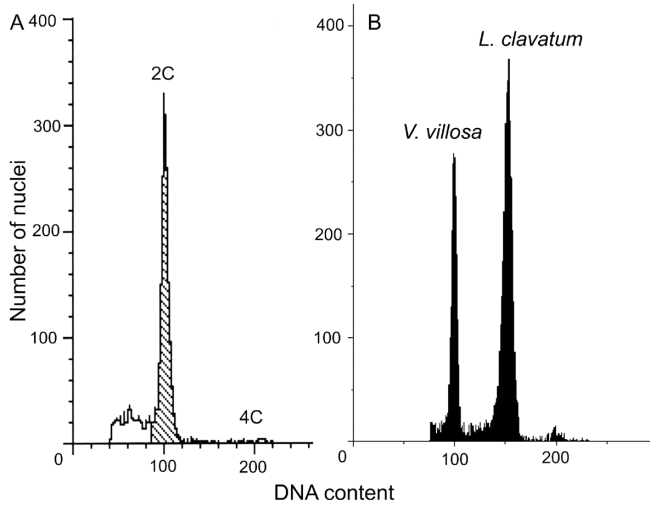

Figure 2 – Histograms of the ploidy level (A) and genome size (B) of *Lycopodium clavatum*.

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