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Endopolyploidy of Endangered Plant Species Ligularia Sibirica in Different Environments

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Abstract. The goal of this study was to detect endopolyploidy of Ligularia sibirica from populations existed in different ecological conditions. This is important step to elaborate the appropriate protection measures of rare and endangered species, which should be based on understanding of ongoing processes in populations. From this point of view the knowledge of genetic diversity, including endopolyploidy level between and within populations, is crucial. L. sibirica is endangered and protected plant species in Latvia which is included in the protected plants list of EU Habitat directive 92/43/EEK Annexes 2 and 4. Perennial herbaceous plant L. sibirica is one of two species of genus Ligularia in Europe. According to the previous data, this species has been disappeared from all previously known locations in Latvia. However, some new locations were found in central part of the country recently. Determination of edopolyploidy level of L. sibirica was performed by the BD FACSJazz® cell sorter (BD Biosciences, USA) with flow cytometer function. In young leafs nine relative fluorescence DNA peaks from 2C up to 64 C were detected. The most common was 2C peak presented in 93% and 63% of samples from Zušu-Staiņu sulphur spring and Krustkalni Nature reserve populations respectively.

Keywords: Ligularia sibirica, flow cytometry, endopolyploidy, endangered species.

I. INTRODUCTION

Ligularia sibirica (L.) Cass. is endangered herbaceous relict species, with restricted population structure throughout the Europe. In Latvia amount of species localities have dramatically decreased during last century. *L. sibirica* is included in the Annex II of Habitats Directive of the Council of European Communities [1]. Typical habitats of *L. sibirica* are wet meadows, springs and spring mires. Changes on chromosome level, for example endopolyploidy, can reflect adaptation under pressure of different stress conditions. Knowledge of the variation of endopolyloidy of individuals of endangered species is important for understanding of ongoing processes in populations. Endopolyploidy is result of the exponential replication of nuclear DNA in the absence of mitosis mainly due the endoreduplication which occur in 90% of all angiosperms [2]. Plant endopolyploidisation is associated with cell differentiation and metabolic activity and is important for normal organ and tissues growth and development [3, 4]. Endoreduplication consist of repeated endocycles without occurrence of mitosis, and chromosomes segregation. Endopolyploidisation leads to presence of various ploidy levels (2C, 4C, 8C...) in the same organism [3]. Many study have reported endopolyploidy in different organism groups [5] mainly focused on plants [6-11], most of information on endopolyploidisation was obtained using experimental data from *Arabidopsis thaliana* [12]. Endopolyploidy can be modulated by many environmental stress factors, including light [3], nutrient availability [12, 13], temperature [10], heavy metal pollution [14], drought and cold stress [2] by activity changes of different molecular processes. The goal of this study was to determine endopolyploidy occurrence in young leafs of *L. sibirica* from two different habitats.

II. MATERIALS AND METHODS

In 2015, *L. sibirica* young leafs (one from each individual) were collected in two localities of the Vidzeme region of Latvia: Zušu-Staiņu sulphur spring and Krustkalni Nature reserve (Figure 1).

Fig. 1. *Ligularia sibirica* habitats in Latvia, 1 - Zušu-Staiņu sulphur mires, 2 - Krustkalni Nature reserve

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Altogether 51 leafs samples were collected: form 22 specimens in Krustkalni and 29 in Zušu-Staiņu sulphur spring. Leaf were dried and kept till analysis in silica gel. Samples for flow cytometry were prepared with a DNA staining kit (Sysmex Partec, PI Absolute, GmbH, Germany) according to manufacturer protocol with some modifications. For each sample, approximately 50 mg of dry leaf material was excised and placed into a glass Petri dish (60 x 15 mm). Dry material was chopped in 500 µL of +4C° cold extraction buffer. For removal of cell fragments the suspension was filtered through 40 µm filter (Falcon, USA) into a 5 mL polypropylene cytometry tube (Falcon, USA), and 1.5 mL of staining buffer was added. Cells nuclei were stained with 10 µL propidium iodide, and incubated in the dark for 24h at +4C° before analysis by flow cytometry. BD FACSJazz® cell sorter (BD Biosciences, USA) with flow cytometer function was used to detect DNA content (C value) of *L. sibirica*. The device was equipped with 100 µm nozzle and used phosphate-buffered saline (BD PharmingenTM PBS, BD Biosciences, USA) as a sheath fluid. Cell counting events were triggered by forward - scattered signal. The excitation of the cell fluorescence was made by 488 nm Coherent Sapphire Solid State (blue) laser. Before measurements, flow cytometer was calibrated using SpheroTM rainbow calibration particles (3.0 –3.4 µm, BD Biosciences, USA) in phosphate buffered saline (PBS). The calibration was considered as successful if the coefficient of variance (CV) of the calibration particles relative fluorescence did not exceed 3%.

Soil samples from both *L. sibirica* grow habitats were collected for laboratory analysis. The soil samples were taken from the root zone near *L. sibirica* plants to 20 cm depth. For each sample at least five sub-samples were collected and mixed into a single sample. Collected soil samples were air-dried and sieved through 2-mm sieve. To determine the plant available amounts of 3 essential nutrients (N, P, K) the soil samples were extracted with 1 M HCl solution (soil/extract volume ratio1:5). The levels of N, P were analyzed by the colorimetry, and K with the flame photometer (Jenwaey PFP7) [17].

III. RESULTS AND DISCUSSION

Flow cytometry analysis of DNA content in *L. sibirica* young leafs from different environments revealed presence of nine relative fluorescence peaks from 2C up to 64 C (Figure 1). Analysis samples from both localities shown that 83% of them were endopolyploid, only 9 samples had cells with one ploidy level: 5 in Zušu-Staiņu locality (Table 2), and 4 in Krustkalni (Table 3). In Zuši-Staiņu locality 93% of the young leafs were found to have 2C DNA content, 34.5% were 4C, 44% were 8C and 34.5% were found to have 16C DNA content. In contrast, in Krustkalni, 2C DNA content was observed only in 63% of samples. Because cell development process in young leafs are still in progress, determination of dominant C value in specimen was performed to prevent G2 phase influence on evaluation of endopopyploidy level. Paying attention to 4C, 8C, 16C, 32C and 64C DNA content peaks that represent DNA amplification by several endocycles in endoreduplication process, in both populations percentage of dominant C value are approximately equal, except 8C which frequency is about 17% higher in Zuši-Staiņu population. Percentage of 64C DNA nucleus among all samples was very low, represented by only 4%. 3C and 10C DNA peaks were observed in only several specimens, and 10C peak were found only in Zuši-Staiņu population. From the point of view of different environments, Zušu-Staiņu population exhibit much higher nutrient degree (Table 1).

Table 1.
Soil Nutrient Composition In Investigated Localities

Locality	Nr. of soil sample	N	P	K
Zušu-Staiņu	1.	250	174	104
	2.	220	92	127
	3.	183	126	135
	4.	88	61	116
	5.	54	22	55
	Mean:	159	95	107.4
Krustkalni	1.	101	65	145
	2.	71	52	47
	3.	85	47	89
	4.	71	41	88
	5.	65	29	65
	6.	76	41	55
	Mean:	78.2	45.8	81.5

Data showed that low contamination of nitrogen is crucial for *L. sibirica* population existence and growth [15], thus high level of nitrogen and other nutrients can be observed as stress factor with negative effect to population existence. It is also possible that nutrients availability just like other environmental factors can affect endopolyploidy level [13, 16]. It is also known, that high levels of endopolyploidy occur in cells with increased secretory function [2]. In this case, it is possible that *L. sibirica* individuals from Zušu-Staiņu habitat undergo adaptation increasing endopolyploidy in their leaf cells during plant development.

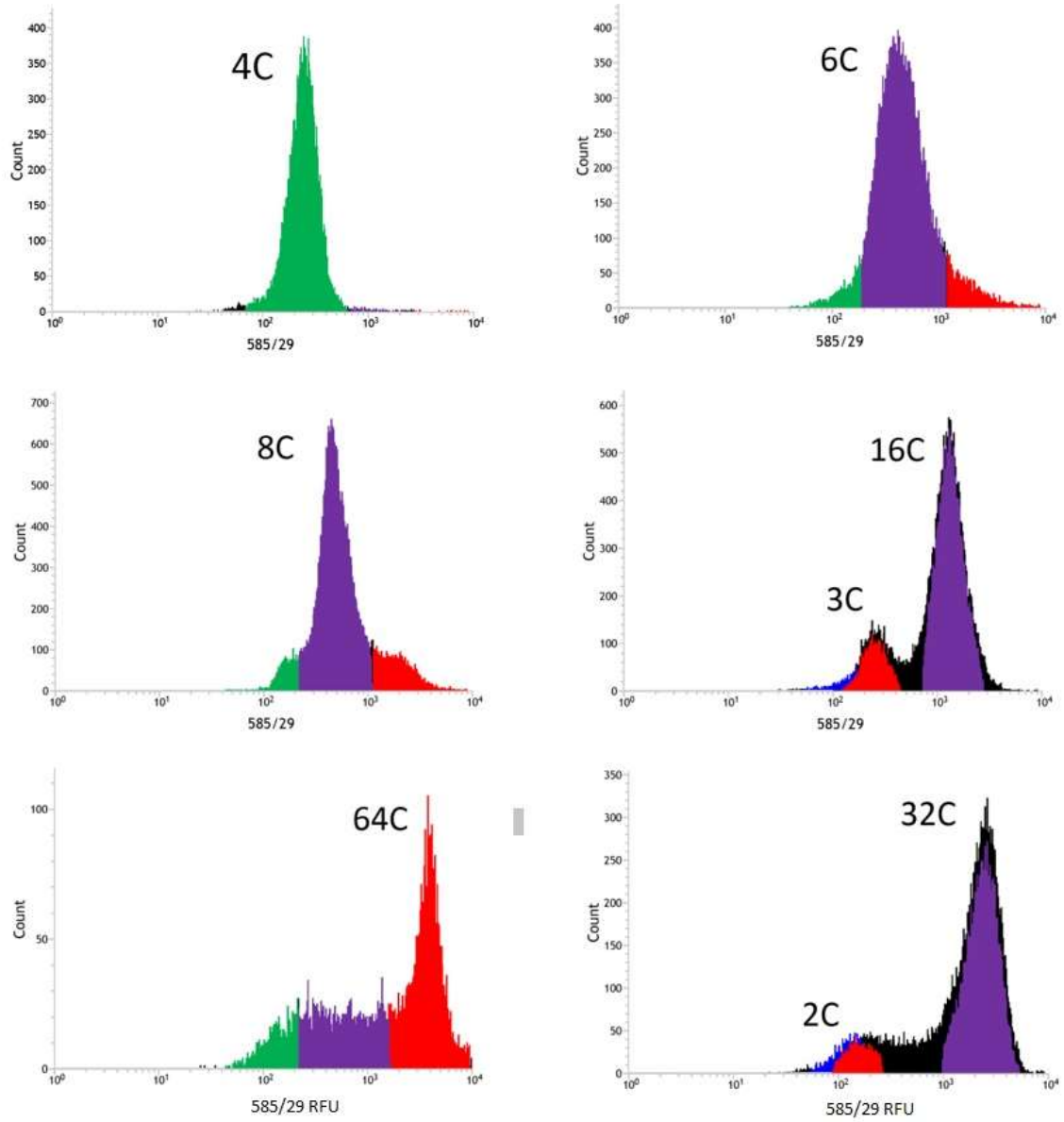

Fig. 2. Density plot showing the relative fluorescence of Ligularia sibirica nuclei staining with PI. Abscissa: RFU (relative fluorescence units) in logarithmic scale at 585 nm; ordinate: number of nuclei.

Table 2.
C Value Peaks of Ligularia Sibirica from Krustkalni Nature Reserve

Nr.	C value	Dominant C value
1	2C + 32C	32C
2	2C + 16C	2C, 16C
3	3C + 16C	3C, 16C
4	2C + 4C + 16C+ >16C	16C
5	2C + 4C + 8C + 16C	8C
6	2C + 32C	32C
7	2C + 32C+ >32C	32C
8	2C + 32C	32C
9	2C + 16C	16C
10	2C + 64C + > 64C	64C

Table 3.
C Value Peaks of Ligularia Sibirica from Zušu-Staiņu Sulphur Spring

11	2C + 4C	2C, 4C
12	4C + 8C	4C
13	4C + 16C	16C
14	2C+ 8C+ 16C	16C
15	4C + 16C + >16C	16C
16	8C	8C
17	2C + 8C	2C, 8C
18	2C + 8C + >8C	2C, 8C
19	4C	4C
20	2C + 32C + >32C	32C
21	2C	2C
22	2C	2C

Nr.	C value	Dominan C value
1	2C + 4C + 8C	2C, 8C
2	2C + 8C	2C, 8C
3	2C+ 4C + 16C	16C
4	2C + 4C + 16C + 32C	32C
5	2C +8C	8C
6	2C +8C	8C
7	4C	4C
8	2C + 8C + >8C	2C, 8C
9	2C + 10C + 16C	10C
10	2C + 8C + 10C	8C, 10C
11	2C + 32C	32C
12	2C + 4C + 8C	8C
13	2C + 8C+ 10C	10C
14	2C + 16C + >16C	16C
15	2C + 16C	2C
16	2C + 8C + 16C	16C
17	2C + 4C	4C
18	2C +8C+ 16C	2C, 8C
19	2C + 8C +> 16C	8C
20	2C + 4C + 64C	64C
21	2C+ 4C	4C
22	2C + 4C + 8C + 10C	4C, 8C
23	2C + 8C	8C
24	2C + 4C + >16C	2C
25	6C	6C
26	2C + 8C + 10C + 16C	10C
27	2C	2C
28	2C	2C
29	2C	2C

IV. CONCLUSION

Flow cytometry was successfully used to determine endoplolyploidy level in *Ligularia sibirica* populations from different localities. Zušu-Staiņu sulphur spring population showing higher level of nutrient contamination what probably is associated with nearness of farmland, revealed especially high percentage of 8C, but not in higher C value peaks. Both populations exhibit different endopolyploidy specifity what is related to pressure of different stress factors. Thus, it can be concluded that nutrient contamination is not the only factor affecting endopolyploidisation in *L. sibirica* young leafs.

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