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## Nuclear DNA content and phenotypic traits of the *Prunus* rootstocks from Poland's gene resources

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### Abstract

The ploidy level can be useful in evaluating reproductive and somatic compatibility, an important parameter in scion and rootstock breeding programs. Evaluation of nuclear DNA content was performed in order to determine ploidy level of 31 rootstocks of genus *Prunus* for plum, sour cherry and sweet cherry. In the first stage of the research, the conditions of flow cytometry analysis with application of propidium iodide for DNA staining were optimized. Repeatable results and relatively good-quality histograms were obtained using Partec extraction buffer with 1% PVP (polyvinylpyrrolidone) addition, with incubation time from 50 to 90 min. The best internal standard for flow cytometry analysis proved to be tomato (*Solanum lycopersicum* L.)  $2C = 1.96$  pg for rootstocks with a nuclear DNA content from 0.55 to 1.64 pg, and soybean (*Glycine max* (L.) Merr.) 2.50 pg for rootstocks with a larger nuclear DNA contents from 1.8 to 2.3 pg. In five plum rootstocks,  $2C$  values ranged from 0.61 to 0.67 pg indicating their diploid chromosome number. One rootstock was identified as tetraploid ( $2C = 1.34$  pg), and four rootstocks as hexaploids owing to their DNA contents from 2.07 to 2.23 pg. One rootstock was considered a pentaploid due to its  $2C$  value of 1.64 pg which was approximately two and a half times more than in diploid *Prunus* sp. In nine cherry rootstocks,  $2C$  DNA values ranged from 0.74 to 0.86 pg indicating their diploid chromosome number. Nine cherry rootstocks were identified as triploids with their  $2C$  DNA contents from 1.03 to 1.24 pg. Two cherry rootstocks were considered as tetraploids having  $2C$  DNA of 1.39 pg. The ploidy level of cherry and plum rootstocks was evaluated in relation to morphological and agronomic traits. The tendency to increase the size of stomata and leaves along with an increase in the ploidy level was observed within *Prunus* rootstock genotypes; however, the correlations between these traits were not so evident. Therefore, stomata and leaf size cannot be considered as a morphological marker indicating ploidy level. The evaluated nuclear DNA contents / ploidy levels as well as stomata and leaf size are the additional descriptors of *Prunus* rootstocks that can be useful for identifying genotypes.

Key words: flow cytometry, hexaploids, pentaploids, ploidy level, plum, cherry rootstocks, tetraploids, triploids.

### Introduction

It is well known that within one genus, the species and even cultivars of the same species differ in the content of nuclear DNA which translates into a ploidy level. It is believed that nuclear DNA content is a specific feature and its assessment by flow cytometry can be helpful in differentiating taxa. For many *Prunus* genotypes used as rootstocks for plum and cherry trees collected in gene resources of the Research Institute of Horticulture in Skierniewice, Poland, the nuclear DNA content is unknown. There are over 400 to 430 *Prunus* species, of which approximately one hundred is important for horticulture and breeding (Das et al., 2011). The species are grouped into four subgenera: *Prunus*, *Cerasus*, *Amygdalus* and *Emplectocladus* (Angiosperm Phylogeny Group, 2009). The main *Prunus* crops are peach, plum, apricot, cherry, almond and their various hybrids, e.g., plumcot. The *Prunus* taxa are mainly cultivated as fruit crops and several species are grown as ornamental plants.

*Prunus* crops like many other fruit crops are propagated by grafting and grown on rootstocks. Grafting onto rootstocks enables the grower to determine the tree's eventual size and improve environmental adaptability, such as tolerance to wet / dry soil conditions, acidity / alkalinity of soil or even hot/cold air temperature and resistance to various pests and diseases (Das et al., 2011).

The basic chromosome number in *Prunus* is  $x = 8$  (Das et al., 2011). Somatic chromosome number of various *Prunus* species varies from diploid to hexaploid (Table 1). Most of the species are diploid ( $2n = 2x = 16$ ), e.g., *P. dulcis*, *P. armeniaca*, *P. avium*, *P. canescens*, *P. cerasifera*, *P. lannesiana*, *P. mahaleb*, *P. munsoniana*, *P. persica*, *P. pumila* and *P. tomentosa*. Some of these species (*P. avium*, *P. cerasifera* and *P. spinosa*) occur in different polyploid forms. There are also several tetraploids ( $2n = 4x = 32$ ) such as *P. cerasus*, *P. fruticosa* and *P. maackii*, and hexaploids ( $2n = 6x = 48$ ) such as

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*P. domestica* and *P. domestica* var. *insititia*. Some of these *Prunus* species have been used for selecting improved rootstock genotypes or served as parental genotypes for creation of hybrid rootstock cultivars combining desired traits (Das et al., 2011).

At present, many rootstocks with different vigour and behaviour are available for plum, sour cherry and sweet cherry trees. The wide genetic diversity and adaptability of rootstocks allow different vegetative-productive behaviours in an orchard. Effects of rootstock on the tree growth, tree productivity and fruit quality of different stone fruit crops was reported in numerous publications (Murri et al., 2013; Sitarek, 2017; Usenik et al., 2017).

The natural polyploid formation within the genus *Prunus* results from both somatic chromosome doubling and union of unreduced gametes with the latter considered most important polyploidisation mechanism (Ramsey, Schemske, 1998; Wang et al., 2018).

Since the nuclear DNA content and ploidy level of many *Prunus* rootstocks are unknown, the evaluation of these traits was performed in order to provide information for germplasm conservation and breeding. In this study, 2C DNA value (somatic nuclear DNA content) / ploidy level of *Prunus* rootstocks was evaluated in relation to stomata and leaf size as well as agronomic trait such as growth vigour. If the size of stomata and leaves and vigour were positively correlated with the level of ploidy, these features could be used as easy-to-use ploidy markers. Such knowledge would also be helpful in selecting parents for crosses in order to obtain hybrids with specific traits, e.g., dwarfing or semi dwarfing effect on tree vigour.

## Materials and methods

**Plant material.** Studies were carried out in 2017–2018 in the Research Institute of Horticulture, Skierniewice, Poland. Thirty one *Prunus* genotypes, 11 rootstocks for plum trees and 20 rootstocks for cherry and sweet cherry, gathered in genetic resources of the Research Institute of Horticulture were used for the research.

**Evaluation of nuclear DNA content.** In the first stage of the research, the conditions of flow cytometry (FCM) analysis with application of propidium iodide (PI) for DNA staining FCM were optimized: type of buffer for nuclei extraction and incubation time (from 15 to 120 min) as well as selection of internal standards (plant genotypes with known nuclear DNA content). Analysis of genome size was done using FCM. Samples were taken in mid-July from six leaves collected randomly from one plant of each genotype. Leaf tissue (0.5–1 cm<sup>2</sup>) was chopped together with a piece (0.5–1 cm<sup>2</sup>) of plant internal standard in a Petri dish in 1.5 mL nuclei isolation Galbraith's buffer (Galbraith et al., 1983) or Partec buffer (Sysmex Partec GmbH, Germany) with slight modification according to Śliwińska (2008) to which propidium iodide (50 µg mL<sup>-1</sup>), RNase (50 µg mL<sup>-1</sup>) and 1% PVP (polyvinylpyrrolidone) were added. As the internal standards, the young leaves of tomato (*Solanum lycopersicum* L.) 'Stupicke' were used (2C DNA = 1.96 pg (Doležel et al., 1992), soybean (*Glycine max* L. Merr.) 'Polanka' 2C DNA = 2.50 pg (Doležel et al., 1994) or maize (*Zea mays* L.) CE-777 2C DNA = 5.43 pg (Lysak, Doležel, 1998) were used. The seeds of reference plants were kindly provided by Institute of Experimental Botany, Czech Republic. Optimization of FCM analysis was performed with reference plants of *Prunus* genotypes (external standards) with the known chromosome

numbers: diploid *P. cerasifera* var. *divaricata* Led. 'Anna' (myrobalan) (2n = 2x = 16), triploid sweet cherry rootstock GiSelA 3 (*P. cerasus* 'Schattenmorelle' × *P. canescens*) (2n = 3x = 24) and hexaploid *P. domestica* 'Eruni' (2n = 6x = 48).

After adding 1.5 mL of the isolation buffer, the samples were filtered through a 30 µm filter and incubated for 50–60 min in room temperature. The fluorescence of the nuclei was measured using analyser CyFlow Ploidy (Sysmex Partec) with an Nd-YAG green laser at 532 nm. Data were analysed by means of software *CyView* (Sysmex Partec). The 2C DNA content of a sample was calculated as the sample peak mean divided by the mean of the standard plant peak and multiplied by the amount of DNA of the standard plant. Samples with at least 5000 nuclei were measured for six leaves of each plant with two runs from each nuclei isolation extract. The ploidy level of rootstocks was evaluated in relation to stomata and leaf size and vigour. The latter feature was classified as weak, intermediate vigour, vigorous or very vigorous, depending on the impact of the rootstock on the tree growth in the orchard, described according to the literature (Murri et al., 2013; Sitarek, 2017; Usenik et al., 2017).

In Table 1, the nuclear 2C DNA contents and/or ploidy levels according to different authors are presented for the *Prunus* species which are present in pedigree of the rootstocks for plums and cherries evaluated in our study. This information was helpful in indicating ploidy levels of *Prunus* genotypes based on FCM analysis and was also used in discussion.

**Phenotype evaluation.** Leaves were collected in late May and June. Leaf area was measured for randomly collected 30 leaves, with an optical planimeter Area Meter AM 350 (ADC Bioscientific, UK). The leaves for measurement of stomata length were collected in mid-June. The abaxial epidermis was isolated from the middle part of the leaves with a transparent adhesive tape and stained with toluidine blue and mounted on slides for microscopic observations according to the procedure of Dyki and Habdas (1996). The stomata measurements were determined for five leaves (×20 stomata) of each genotype using a Nikon Eclipse 80i microscope with the program NIS-Elements BR 2.30 (Nikon Instruments Europe BV, The Netherlands) at 400× magnification.

**Statistical analysis.** Data of all parameters were analysed with analysis of variance (ANOVA)-nested design. All calculations were done with the package *Statistica*, version 10 (StatSoft Inc., USA). The means were compared by Tukey's test at  $p = 0.05$ .

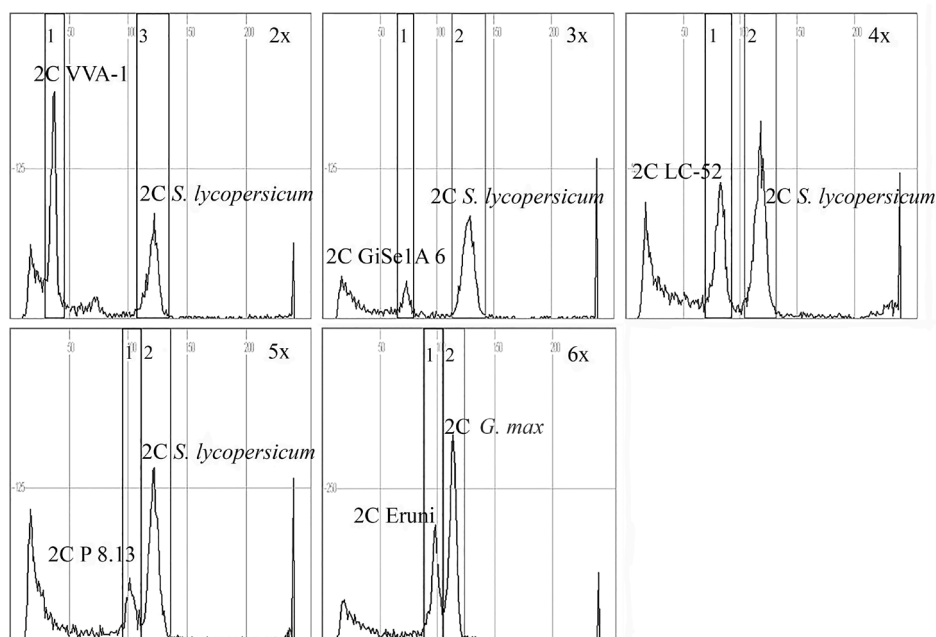
## Results

Repeatable results and relatively good-quality histograms were obtained using Partec extraction buffer with 1% PVP addition, with incubation time from 50 to 130 min. The best internal standard for FCM analysis of the rootstocks for plums and cherries with a nuclear DNA content from about 0.7 to 1.4 pg proved to be *Solanum lycopersicum* (2C = 1.96 pg), of which peaks 2C and 4C did not coincide with the peaks of the genotypes tested (Fig. 1). However, peaks of *S. lycopersicum* overlapped partially with the peaks of *Prunus* genotypes with larger genomes tested. For rootstock genotypes with a nuclear DNA contents from 1.8 to 2.3 pg, *Glycine max* (2.50 pg) was selected as the internal standard. In the second stage, the optimized method and well-chosen internal standards allowed to assess with high precision the nuclear DNA content / ploidy level of 31 *Prunus* genotypes of the rootstocks for plums and cherries.

**Table 1.** Ploidy levels and nuclear DNA contents of reference *Prunus* genotypes, which are the parental genotypes for rootstock for plum, cherry and sweet cherry trees

Genotype	Ploidy level	References for ploidy level	Nuclear 2C DNA content (pg), references	References for nuclear DNA content*
<i>P. amygdalus</i> Batsch, syn. <i>Prunus dulcis</i> , almond	2×	B, D, Pr	0.66	B
<i>P. armeniaca</i> L., apricot	2×	Dic, D, Pr	0.61, 0.60	Ar, Dic, Di
<i>P. avium</i> (L.) L., sweet cherry	2×, 3×, 4×	Dic, D, Pr, W	0.70, 0.67	Ar, Dic, Di
<i>P. canescens</i> Bois, hoary cherry	2×	D, Pr		
<i>P. cerasifera</i> , cherry plum	2×, 2× + 1, 3×, 4×, 6×	D		
<i>P. cerasus</i> L., sour cherry	4×	Dic, D, Pr	1.24, 1.36–1.42	Ar, Dic, Di
<i>P. domestica</i> L., plum	6×	Dic, D, Z	1.83, 1.83, 1.86, 2.10	Ar, Dic, Di, Z
<i>P. domestica</i> ssp. <i>insititia</i> L., damson	3×, 6×	D, BT, Pr, Z	2.14, 1.99–2.13	Z, BT
<i>P. fruticosa</i> Pall., European dwarf cherry	4×	D, Pr		
<i>P. incisa</i> Thunb., Fuji cherry	2×	Pr		
<i>P. maackii</i> , manchurian cherry	4×	P, Pr	0.96–0.97	P
<i>P. lannesiana</i> (Carriere) E.H. Wilson, syn. <i>P. serrulata</i> var. <i>lannesiana</i> , Japanese cherry	2×, 3×	Pr, W		
<i>P. mahaleb</i> L., mahaleb cherry	2×	D, Pr		
<i>P. munsoniana</i> , W.Wight & Hedrick, wild-goose plum	2×	E, Pr		
<i>P. persica</i> (L.) Batsch, peach	2×	Dic, D, Pr	0.54–0.55	Ar, Dic, Di
	3× (polyploidisation)		0.58–0.64	B
	4× (polyploidisation)		0.98	B
<i>P. pseudocerasus</i> Lindl., Cambridge cherry	4×, 5×, 6×	G, W		
<i>P. pumila</i> L., sand cherry	2×	Pr		
<i>P. salicina</i> Lindl., Japanese plum	2×, 4×	D, BT, Pr, W	0.44–0.97	BT
<i>P. spinosa</i> L., blackthorn	2×, 3×, 4×, 5×, 6×	D, O, Pr, Z	1.40	Z
<i>P. tomentosa</i> Thunb., nanking cherry	2×	D, W	0.57	B
GiSelA	3×	V		

\* – the letters in columns indicate references for ploidy levels or nuclear DNA contents: Ar – Arumuganathan, Earle (1991), B – Baird et al. (1994), BT – Ben Tamarzizt et al. (2015), D – Das et al. (2011), Dic – Dickson et al. (1992), Di – Dirlwanger et al. (2009), G – Gu et al. (2014), O – Okie, Weinberger (1996), P – Pooler et al. (2012), Pr – *Prunus* Vulnerability Statement (2017), V – Vujović et al. (2012), W – Wang et al. (2018), Z – Žabka et al. (2018).



2× – diploid plum rootstock VVA-1, 3× – triploid cherry rootstock GiSelA 6, 4× – tetraploid cherry rootstock LC-52, 5× – pentaploid plum rootstock ‘Mariana P 8.13’, 6× – hexaploid plum rootstock ‘Eruni’

**Figure 1.** Histograms of nuclear DNA / ploidy level estimation using flow cytometry (FCM) with internal standards: *Solanum lycopersicum* (2C DNA = 1.96 pg) for the taxa of nuclear DNA content below 1.70 pg and *Glycine max* (2C DNA = 2.50 pg) for hexaploid genomes (2.07–2.24 pg)

For the reference *Prunus* genotypes of the known chromosome number, 2C DNA values were 0.68 pg for diploid *P. cerasifera* var. *divaricata* Led. 'Anna', 1.18 pg for triploid GiSelA 3 (*P. cerasus* 'Schattenmorelle' × *P. canescens*) and 2.16 pg for hexaploid *P. domestica* 'Eruni' (Table 2). In four plum rootstock genotypes ('Ferciana Ishtara', VVA-1, 'Druzba' and GF 667), 2C values ranged from 0.61 to 0.67 pg indicating their diploid chromosome number. One rootstock 'Fereley Jaspi' was identified as tetraploid (2C = 1.34 pg) and three rootstocks as hexaploids ('St. Julien A', 'Pixy' and GF 655/2) owing to their DNA contents from 2.07 to 2.23 pg. The rootstock 'Mariana P 8.13' was considered pentaploid due to its 2C value of 1.64 pg, which was

approximately two and a half times more than in diploid *Prunus* sp. (2C DNA = 0.66 pg).

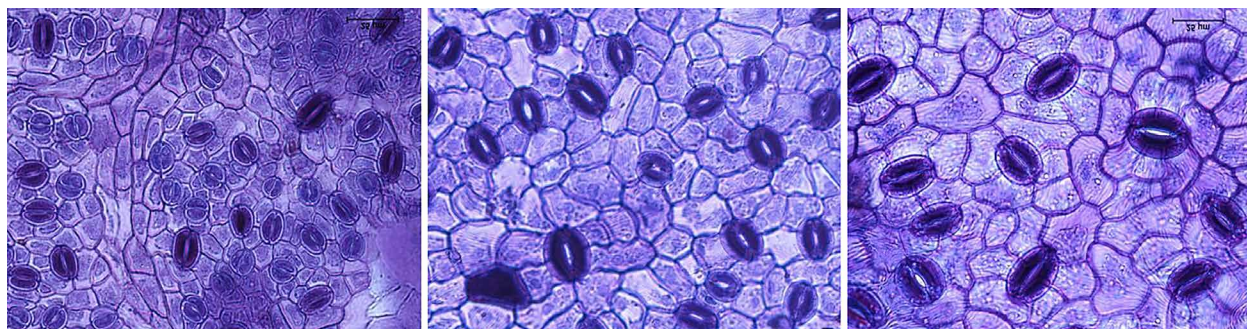
In nine rootstocks for cherry trees: F 12/1, PiKu 1, Piku 3, INRA SL 64, 'Maxma Delbard 14 Brokforest', 'Ferci SL 405', GM 79, LC-13 and L-2, 2C DNA values ranged from 0.55 to 0.86 pg indicating their diploid chromosome number (Table 2). Nine rootstocks were identified as triploids: 'Colt', GiSelA 3, GiSelA 5, GiSelA 6, P-HL A, P-HL C, PiKu 4, VSL-1 and VSL-2, with their 2C DNA contents from 1.03 to 1.24 pg. Two rootstocks were considered tetraploid: WC-13 and LC-52, having 2C DNA of 1.39 pg.

Genotypes differ significantly in the leaf size and stomata length (Tables 2–3, Figs 2–3). Within rootstocks

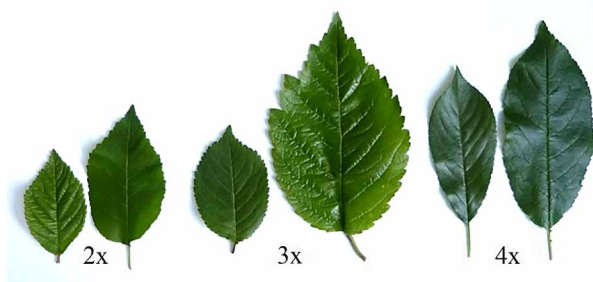
**Table 2.** Nuclear DNA contents and ploidy levels of *Prunus* genotype rootstocks for plum, sour cherry and sweet cherry trees in relation to the leaf area, stomata length and vigour level

Genotype	Parentage	Nuclear 2C DNA content pg	Ploidy level	Leaf area cm <sup>2</sup>	Stomata length μm	Vigour level
Rootstocks for plum trees						
VVA-1 (Krymsk 1)	<i>P. tomentosa</i> × <i>P. cerasifera</i>	0.61 ± 0.003	2×	16.3 d-c	24.1 ± 2.38 d	intermediate vigour
Druzba	<i>P. pumila</i> × <i>P. armeniaca</i>	0.65 ± 0.006	2×	15.2 d-g	22.1 ± 2.39 e	very vigorous
Ferciana Ishtara	<i>P. salicina</i> 'Belsiana' × ( <i>P. cerasifera</i> × <i>P. persica</i> )	0.67 ± 0.013	2×	20.9 cd	21.4 ± 2.98 e	vigorous
GF 677	( <i>P. persica</i> × <i>P. amygdalus</i> )	0.67 ± 0.000	2×	26.3 ab	30.2 ± 3.08 a	vigorous
Myrobalan	<i>P. cerasifera</i> var. <i>divaricata</i>	0.68 ± 0.005	2×	11.4 g	15.9 ± 3.32 g	very vigorous
Fereley Jaspi	<i>P. salicina</i> 'Methley' × <i>P. spinosa</i>	1.34 ± 0.017	4×	14.8 fg	21.3 ± 2.43 e	vigorous
Mariana P 8.13	<i>P. cerasifera</i> × <i>P. munsoniana</i>	1.64 ± 0.039	5×	20.1 d	19.6 ± 1.99 f	very vigorous
Pixy	<i>P. insititia</i>	2.07 ± 0.250	6×	22.4 bc	26.8 ± 2.64 c	weak
Eruni	<i>P. domestica</i>	2.16 ± 0.057	6×	30.8 a	21.7 ± 2.81 e	intermediate vigour
GF 655/2	<i>P. insititia</i>	2.21 ± 0.029	6×	19.9 c-e	28.3 ± 2.54 b	intermediate vigour
St. Julien A	<i>P. insititia</i>	2.23 ± 0.017	6×	19.3 c-f	24.8 ± 2.50 d	intermediate vigour
Rootstocks for sour cherry and sweet cherry trees						
LC-13	<i>P. avium</i> × <i>P. cerasus</i>	0.55 ± 0.008	2×	13.3 k	18.9 ± 2.98 kl	intermediate vigour
Ferci SL 405	<i>P. mahaleb</i>	0.75 ± 0.013	2×	18.9 h-k	25.7 ± 3.36 c-e	vigorous
INRA SL 64	<i>P. mahaleb</i>	0.75 ± 0.006	2×	19.7 h-k	25.2 ± 2.39 d-f	vigorous
L-2 (Krymsk 7)	<i>P. lannesiana</i>	0.77 ± 0.010	2×	15.9 jk	23.7 ± 2.02 gh	intermediate vigour
PiKu 3 (4.83)	<i>P. pseudocerasus</i> × ( <i>P. canescens</i> × <i>P. incisa</i> )	0.77 ± 0.010	2×	21.1 h-j	24.9 ± 2.45 d-g	vigorous
Maxma Delbard 14 Brokforest	<i>P. avium</i> × <i>P. mahaleb</i>	0.79 ± 0.006	2×	32.2 d-f	24.1 ± 2.59 f-h	vigorous
GM 79 (Camil)	<i>P. canescens</i>	0.80 ± 0.021	2×	33.6 de	20.1 ± 2.34 k	intermediate vigour
PiKu 1 (4.20)	<i>P. avium</i> × ( <i>P. canescens</i> × <i>P. tomentosa</i> )	0.81 ± 0.008	2×	20.2 h-k	23.4 ± 2.31 hi	weak
F 12/1	<i>P. avium</i>	0.86 ± 0.013	2×	45.3 b	22.3 ± 2.07 ij	very vigorous
Colt	<i>P. avium</i> (F299/2) × <i>P. pseudocerasus</i>	1.03 ± 0.039	3×	57.8 a	24.5 ± 2.88 e-h	very vigorous
PiKu 4 (4.22)	( <i>P. canescens</i> × <i>P. tomentosa</i> ) × <i>P. avium</i>	1.12 ± 0.022	3×	31.6 d-f	22.2 ± 2.39 ij	intermediate vigour
VSL-1	<i>P. fruticosa</i> × <i>P. lannesiana</i>	1.12 ± 0.029	3×	22.3 g-i	25.0 ± 2.79 d-f	intermediate vigour
VSL-2 (Krymsk 5)	<i>P. fruticosa</i> × <i>P. lannesiana</i>	1.12 ± 0.029	3×	15.1 jk	26.1 ± 3.16 cd	vigorous
GiSelA 5 (146/2)	<i>P. cerasus</i> 'Schattenmorelle' × <i>P. canescens</i>	1.13 ± 0.017	3×	29.4 d-f	21.8 ± 2.45 j	intermediate vigour
GiSelA 6 (148/1)	<i>P. cerasus</i> 'Schattenmorelle' × <i>P. canescens</i>	1.14 ± 0.005	3×	29.0 e-g	27.0 ± 3.58 bc	intermediate vigour
GiSelA 3 (209/1)	<i>P. cerasus</i> 'Schattenmorelle' × <i>P. canescens</i>	1.18 ± 0.005	3×	21.0 h-j	26.2 ± 2.54 cd	weak
P-HL A (84)	<i>P. avium</i> × <i>P. cerasus</i>	1.20 ± 0.015	3×	41.9 bc	25.7 ± 2.91 c-e	intermediate vigour
P-HL C (6)	( <i>P. avium</i> × <i>P. cerasus</i> )	1.24 ± 0.037	3×	48.3 b	25.2 ± 2.52 d-f	weak
LC-52 (Krymsk 6)	<i>P. cerasus</i> 'Lyubskaya' × ( <i>P. cerasus</i> 'Michurin' × <i>P. maackii</i> )	1.39 ± 0.010	4×	25.6 fg	30.8 ± 3.02 b	intermediate vigour
WC-13	<i>P. cerasus</i> 'Vladimirskaia' × ( <i>P. cerasus</i> 'Michurin' × <i>P. maackii</i> )	1.39 ± 0.012	4×	36.7 cd	27.8 ± 3.02 b	intermediate vigour

Note. The mean separation within columns by Tukey's test at  $p = 0.05$ ; the means followed by the same letter do not differ at  $p = 0.05$ .



**Figure 2.** Stomata of plum rootstocks, from the left: diploid myrobalan (with very small and the incidental larger stomata), hexaploid 'Eruni' and hexaploid GF 655/2 with large stomata (bar = 25 µm)



**Figure 3.** Leaves of cherry rootstocks: diploids LC-13 and 'Maxma Delbard' (2×), triploids VSL-1 and 'Colt' (3×) and tetraploids LC-53 and WC-13 (4×)

for plums, the smallest stomata with size of 15.9 and 19.6 µm, were recorded for diploid myrobalan and pentaploid 'Mariana P 8.13' and the largest stomata, 28.3 and 30.2 µm, for hexaploid GF 655/2 and diploid GF 677, respectively. The intermediate stomata lengths ranging from 21.3 to 24.1 µm were observed for three diploid, one

tetraploid and one hexaploid plum rootstocks. Within the plum rootstocks of the smallest leaves (11.4–16.3 cm<sup>2</sup>), there were three diploids, including myrobalan, and one tetraploid genotype. Among the plum rootstocks having intermediate and larger leaves (19.3–30.8 cm<sup>2</sup>), there were two diploids, one pentaploid and four hexaploids with the largest leaves recorded for hexaploid 'Eruni'.

Within cherry rootstocks, the correlation of stomata size and ploidy level was more evident (Tables 2–3). Among 10 genotypes of the smaller stomata (18.9–24.9 µm), there were eight diploids and two triploids while within cherry rootstocks of larger stomata (25.0–30.8 µm), there were two diploids, six triploids and two tetraploids. However, the tendency in the increase of leaf size with the increase in the ploidy level was not so clear. The smallest leaves were observed for diploid LC-13 (13.3 cm<sup>2</sup>) and triploid VSL-2 (15.1 cm<sup>2</sup>) while the largest leaves 48.3 and 57.8 cm<sup>2</sup> were found for triploids P-HL C and 'Colt', respectively. In turn, tetraploids WC-13 and LC-52 had leaves of intermediate size.

**Table 3.** Effect of ploidy level on leaf area and stomata length of *Prunus* rootstocks

Rootstocks for plum trees			Rootstocks for sour cherry and sweet cherry trees		
ploidy level	leaf area cm <sup>2</sup>	stomata length µm	ploidy level	leaf area cm <sup>2</sup>	stomata length µm
2×	18.1 bc	22.5 b	2×	24.3 b	23.6 c
4×	14.8 c	21.3 b	3×	32.9 a	24.8 b
5×	20.1 ab	19.6 c	4×	31.2 a	29.3 a
6×	23.1 a	25.4 a			
<i>p</i>	0.00	0.00		0.00	0.00

*Note.* The mean separation within columns by Tukey's test at  $p = 0.05$ ; the means followed by the same letter do not differ at  $p = 0.05$ .

In general, comparison of averages for individual ploidy levels showed a tendency to increase the size of stomata along with an increase in the level of ploidy (Table 3). Such tendency was observed in rootstocks for sweet and sour cherries. In plum rootstocks, only hexaploids had on average significantly larger stomata and leaves compared to other ploidy levels. In rootstocks for cherries, triploid and tetraploid levels had on average significantly larger stomata and leaves compared to diploid level. It should be noted that pentaploids and hexaploids were not detected among cherry rootstocks.

## Discussion

The ploidy level as well as DNA content of *Prunus* sp. was first estimated using FCM analysis by Arumuganathan and Earle (1991). These authors assessed the nuclear DNA amount for six species of *Prunus*,

reporting 2C DNA values 0.54–0.70 pg for diploids, 1.24–1.42 pg for tetraploids and 1.83 pg for hexaploids. Later, Dickson et al. (1992) evaluated 2C DNA of nine *Prunus* species and cultivars, and evaluations of these authors for particular ploidy levels were very similar. The authors mentioned above found that the genome size showed little variation within the diploid species of genus *Prunus* with the smallest value for *P. persica* and the largest for *P. avium*.

In our study, similar variation in 2C DNA values of diploid taxa especially within cherry rootstocks was observed. These values ranged from 0.55 pg for LC-13 to 0.86 pg for F 12/1. Besides, the latter was selected from *P. avium*. And this species was reported to have larger genome compared to other diploid species (Arumuganathan, Earle, 1991; Dickson et al., 1992). Such differences in nuclear DNA contents between cultivars or species possessing the same chromosome number

and belonging to the same genus are widely known in other genera, e.g., *Lilium* (Van Tuyl, Boon, 1997), *Malus* (Korban et al., 2009; Podwyszyńska et al., 2016); *Tulipa* (Zonneveld, 2009) and *Narcissus* (Zonneveld, 2010). The nuclear 2C DNA content is therefore considered as one of the taxa descriptors.

Our analysis showed that F 12/1 selected from *P. avium* had 2C DNA of 0.86 pg while the values reported by other authors (Arumuganathan, Earle, 1991; Dickson et al., 1992) were lower (0.70 pg). Such differences between 2C values obtained for the same species and reported by various authors resulted probably from various FCM methods, in which different buffers and incubation times as well as various DNA staining methods and internal standards were used. It was reported that in *Prunus*, nuclear DNA content evaluation was performed using the following internal standards: chicken erythrocytes of 2C = 2.33 pg (Arumuganathan, Earle, 1991; Dickson et al., 1992; Baird et al., 1994), *Glycine max* 2C = 2.50 pg (Pooler et al., 2012; Ben Tamarziz et al. 2015; Żabka et al., 2018) and *Zea mays* with 2C = 5.43 pg (Jedrzejczyk, Sliwiska, 2010). In our study, *Solanum lycopersicum* of 2C = 1.96 pg was considered the most suitable internal standard for evaluation of ploidy levels from diploid to pentaploid levels of *Prunus* genotypes. Only hexaploid taxa required another internal standard plant, namely *Glycine max*. Thus the peaks for 2C *Prunus* hexaploids overlapped with *S. lycopersicum* 2C pick, both having similar nuclear DNA contents.

In our study, five rootstocks for plum and nine rootstocks for cherries were identified as diploids, which correspond to their origin from diploid parents. Among diploid rootstocks, myrobalan was selected from diploid *P. cerasifera* var. *divaricata* and the cherry rootstocks such as 'Ferci SL 405' and INRA SL 64, L-2, GM 79 and F 12/1 were the selections of the following diploid species: *P. mahaleb*, *P. lannesiana*, *P. canescens* and *P. avium*, respectively. In turn, other diploid cherry rootstocks are generally the hybrids of diploid parental species mentioned above and some other diploids such as *P. incisa*, *P. pseudocerasus* and *P. tomentosa*. Only diploid PiKu 3 has more complicated pedigree, which comprises two diploid and one tetraploid genotypes. In the case of triploid cherry rootstocks, all of them are the hybrids of diploid and tetraploid species, with one exception of PiKu 4, which has in its pedigree three diploid species. In the latter, one of the parent genotypes is *P. avium* for which, except of diploid, also triploid and tetraploid forms are known (Das et al., 2011). On the other hand, PiKu 4 can be a hybrid of only diploid parents where one of them could be a donor of unreduced gamete 2n. Such phenomenon is widespread within *Prunus* genus (Wang et al., 2018).

In the case of plum rootstocks evaluated in our study, their ploidy levels correspond well with their origin. All of the diploid rootstock genotypes were selected from diploid species or are the interspecific hybrids of diploid species. Tetraploid 'Fereley Jaspi' has probably tetraploid parental forms of *P. salicina* and *P. spinose*. In pentaploid plum rootstock 'Mariana P 8.13', probably one of the parents is tetraploid or hexaploid form of *P. cerasifera* since such forms are often found (Das et al., 2011). It has been documented that autopolyploids of *P. pseudocerasus* can produce during meiosis multiple fertile gametes with complete chromosome sets, e.g., 4n,

leading to formation of pentaploids (Ramsey, Schemske, 1998). We suppose that similar phenomenon could occur also in autopolyploid *P. cerasifera*, which, apart from diploid *P. munsoniana*, was the second parent of the plum rootstock P 8.13. In turn, all accessions identified as hexaploids (four plum rootstocks) were selections of hexaploid *P. domestica* or *P. domestica* var. *insititia*.

For genotypes within a species and genus, the higher ploidy level is generally associated with increased sizes of stomata and leaves as, e.g., in tea (Wachira, 1994), banana and plantain (Vandenhout et al., 1995) or mulberry (Laltanmawii, Roychowdhuri, 2010). Although, in *Prunus* rootstocks, comparison of averages for ploidy levels showed a certain tendency to increase the stomata and leaf size along with an increase in the ploidy level; however, the correlations between these traits were not so evident within the genus *Prunus* (plum and cherry rootstocks) as compared to the high positive correlation between nuclear DNA amount and stomatal length found for the cultivars of the species *Malus × domestica* (Tatum et al., 2005; Korban et al., 2009; Podwyszyńska et al., 2016). This probably results from the fact that the genus *Prunus* includes many various species and hybrids, for which phenotypic and genetic variability is much higher compared to variability of the cultivars within one species such, e.g., *Malus × domestica*. Thus, in plum rootstocks, the smallest (19.6 µm) stomata were found in pentaploid (P 8.13) and in contrast, the largest (30.2 µm) stomata were recorded in one of the diploid genotype (GF 677). So stomata and leaf size cannot be considered as a morphological marker indicating ploidy level for *Prunus* rootstocks. Moreover, there is no correlation between ploidy level and vigour of individual rootstocks.

The evaluated nuclear DNA contents / ploidy levels as well as stomata and leaf size are the additional descriptors of *Prunus* rootstock genotypes that can be useful for identifying genotypes. In addition, it is well known that polyploids with an odd number of chromosome sets, e.g., triploids or pentaploids, are low-fertile or infertile (Bharadwaj, 2015). Therefore, the knowledge about the ploidy level of a given *Prunus* genotype can be useful for planning breeding programs. It can be helpful in the selection of parental forms for crosses and allows predicting the reproductive abilities of hybrids.

## Conclusions

1. Genome sizes of 31 *Prunus* of rootstocks for plum and cherry trees were evaluated using flow cytometry (FCM) analysis. In plum rootstocks, five diploid, one tetraploid, one pentaploid and four hexaploid genotypes were detected while in rootstocks for cherry trees, nine diploid, nine triploid and two tetraploid genotypes were identified.

2. The tendency to increase the size of stomata and leaves along with an increase in the ploidy level is observed within *Prunus* rootstocks for cherry trees. In plum rootstocks, only hexaploids have on average significantly larger stomata and leaves compared to other ploidy levels. However, the correlations between these traits are not so evident. Therefore, stomata and leaf size cannot be considered as a morphological marker indicating ploidy level.

3. There is no correlation between ploidy level and vigour of individual rootstocks.

4. The evaluated nuclear DNA contents / ploidy levels as well as stomata and leaf size are the additional, useful descriptors for identifying *Prunus* rootstock genotypes.

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## ***Prunus* genties poskiepių iš Lenkijos genetinių išteklių branduolio DNR kiekis ir fenotipiniai požymiai**

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### **Santrauka**

Ploidiškumo lygis gali būti naudingas vertinant reprodukcinį ir somatinį suderinamumą, kuris yra svarbus parametras įskiepių ir poskiepių selekcinėse programose. Branduolio DNR kiekio įvertinimas buvo atliktas siekiant nustatyti 31 *Prunus* genties (slyvos, vyšnios ir trešnės) vaismedžių ploidiškumą. Pirmajame tyrimo etape buvo optimizuotos srovinės citometrijos analizės sąlygos naudojant propidžio jodidą (PJ) DNR dažymui. Pakartojami rezultatai ir palyginti geros kokybės histogramos buvo gauta naudojant ekstrahavimo buferį, papildytą 1 % polivinilpirolidono (PVP), inkubuojant nuo 50 iki 90 minučių.

Geriausias srovinės citometrijos analizės vidinis standartas poskiepiams, kurių branduolio DNR kiekis yra nuo 0,55 iki 1,64 pg, buvo valgomasis pomidoras (*Solanum lycopersicum* L.)  $2C = 1,96$  pg, o poskiepiams su didesniu branduolinės DNR kiekiu (1,8–2,3 pg) – gauruotoji soja (*Glycine max* (L.) Merr.) 2,50 pg. Penkiuose slyvos poskiepiuose  $2C$  vertės svyravo nuo 0,61 iki 0,67 pg; tai rodė jų diploidinį chromosomų skaičių. Vienas poskiepis buvo identifikuotas kaip tetraploidas ( $2C = 1,34$  pg), keturi poskiepiai – kaip heksaploidai, kurių DNR kiekis buvo nuo 2,07 iki 2,23 pg. Vienas poskiepis identifikuotas kaip pentaploidas dėl jo 1,64 pg  $2C$  vertės; tai buvo apie 2,5 karto daugiau nei diploidinėje *Prunus* sp. Devyniuose vyšnios poskiepiuose  $2C$  DNA vertės svyravo nuo 0,74 iki 0,86 pg; tai rodė jų diploidinį chromosomų skaičių. Devyni vyšnios poskiepiai buvo identifikuoti kaip triploidai, kurių  $2C$  DNR kiekis svyravo nuo 1,03 iki 1,24 pg. Du vyšnios poskiepiai identifikuoti kaip tetraploidai, kurių  $2C$  DNR kiekis buvo 1,39 pg. Vyšnios ir slyvos poskiepių ploidiškumo lygis buvo įvertintas atsižvelgiant į morfologinius ir agronominius požymius. *Prunus* poskiepio genotipuose didėjant ploidiškumo lygiui nustatyta žiotelių ir lapų didėjimo tendencija, tačiau koreliacijos tarp šių požymių nebuvo tokia akivaizdi. Taigi, žiotelių ir lapų dydis negali būti laikomas morfologiniu žymekliu, rodančiu ploidiškumo lygį. Įvertintas branduolinės DNR kiekis / ploidiškumo lygis, taip pat ir žiotelių ir lapų dydis yra papildomi *Prunus* poskiepių deskriptoriai, kurie gali būti naudingi identifikuojant genotipus.

Reikšminiai žodžiai: heksaploidai, pentaploidai, ploidiškumo lygis, slyva, srovinė citometrija, tetraploidai, triploidai, vyšnios poskiepiai.