www.czasopisma.pan.pl PAN www.journals.pan.pl
POLSKA AKADEMIA NAUK

ACTA BIOLOGICA CRACOVIENSIA Series Botanica 58/1: 81–93, 2016
DOI: 10.1515/abcsb-2016-0008

NUCLEAR DNA CONTENT AND PLOIDY LEVEL OF APPLE CULTIVARS INCLUDING POLISH ONES IN RELATION TO SOME MORPHOLOGICAL TRAITS

MAŁGORZATA PODWYSZYŃSKA*, DOROTA KRUCZYŃSKA, ALEKSANDRA MACHLAŃSKA, BARBARA DYKI, IWONA SOWIK

Research Institute of Horticulture, Konstytucji 3 Maja 1/3, 96-100 Skierniewice, Poland

Received February 10, 2016; revision accepted May 6, 2016

Apple species and cultivars differ in nuclear (2C) DNA content and ploidy level. The majority of these genotypes are diploids, but there are some triploids and a few tetraploids. Nuclear DNA content is a specific feature and its flow cytometric evaluation can be helpful in differentiating taxa. For many apple genotypes – including all the Polish ones, these characteristics are not known. 2C DNA was evaluated in relation to leaf, flower, fruit, pollen grain and stomata sizes as well as to the flowering time for seventy genotypes (including 46 Polish cultivars) gathered in the gene bank of the Research Institute of Horticulture, Skierniewice, Poland. For standard cultivars with the known chromosome number, 2C value was 1.71 pg for diploid cultivar 'Alwa' (2n=2x=34), 2.55 pg for triploid 'Boskoop' (3x=51), and 3.37 pg for tetraploid genome (4x=68) of mixoploid 'McIntosh 2x+4x'. In 61 cultivars (including 41 Polish ones), the nuclear DNA content ranged from 1.58 to 1.78 pg indicating their diploid chromosome number. Five cultivars were identified as triploids ('Bursztówka Polska', 'Pagacz', 'Rapa Zielona', 'Rarytas Śląski' and 'Witos') owing to their nuclear DNA amount ranging between 2.42 and 2.58 pg. Leaf, flower, fruit, stomata and pollen grain sizes were on average significantly larger in triploids. Thus, in 3x plants the mean leaf surface was 49.1 cm^2, flower diameter – 52.4 mm, fruit weight – 204.7 g, stomata length – 32.1 μm and pollen grain diameter – 33.7 μm, whereas in diploids – 36.0 cm^2, 46.1 mm, 162.7 g, 28.4 μm and 30.7 μm, respectively. Pollen grain viability was on average significantly higher in diploids (75.6%), compared to triploids (22%). These results confirm that in apple, as in many other plant species, the higher ploidy level of triploids is generally associated with increased sizes of pollen grains, stomata, flowers, fruits and leaves but decreased pollen viability. No clear correlation between ploidy level and flowering time was found. In the case of mixoploid apple genotypes possessing diploid and tetraploid genomes, some phenotype observation is helpful in describing the ploidy level of the histogenic layers, L1 and L2. Small stomata sizes (similar to diploid) indicate diploid L1 and larger leaf sizes, compared to diploid counterparts, show tetraploid L2. The results will be used for breeding, in which it is important to determine maternal and paternal genotypes as well as the direction of the crossing that is of great importance in obtaining seeds and materials for further selection.

Keywords: *Malus* × *domestica*, nuclear DNA content, phenotype, ploidy level, ploidy chimera

INTRODUCTION

Apple species and cultivars differ in the nuclear (2C) DNA content and ploidy level. The majority of these genotypes are diploids (2x=34), but there are some triploids (3x=51) and a few tetraploids (4x=68) (Janick, 1996; Tatum et al., 2005; Korban et al., 2009; Pereira-Lorenzo et al., 2007; Ramos-Cabrer et al., 2007; Höfer and Meister, 2010; Jędrzejczyk and Śliwińska, 2010; Considine et al., 2012; Sedysheva and Gorbacheva, 2013). Triploid apples, generally characterized by their large fruits and other valuable features, have been very attractive to both growers and consumers (Janick, 1996; Sedysheva and Gorbacheva, 2013). The listed authors estimate that 10% of the commonly grown cultivars are triploids which have originated naturally from fertilization of unreduced gametes. While studying the pattern of genome sexual polyploidization in diploid *Malus*, Considine et al. (2012) proved that all triploids were derived from 2n egg cell fertilized with n sperm cell. The authors suggested that triploids can also be useful in the seed crop breeding program and for commercial seed

* Corresponding author, email: Malgorzata.Podwyszynska@inhort.pl

PL ISSN 0001-5296

production as alternative male-sterile materials since sperm cells of triploids are reportedly weakly fertile or sterile but their egg cells are usually fertile (Janick et al., 1996; Ramanna and Jacobsen, 2003; Dzialuk et al., 2007). Polyploidy and aneuploidy are important for horticultural crops; they usually express valuable traits in tree architecture or biological styles, for example dwarfing tree-systems as in cherry rootstock (Webster, 1996) and citrus hybrid trees (Jaskani and Khan, 2000).

It is known that within one genera, the species of plants and even their varieties can differ in terms of nuclear DNA content in spite of an equal number of chromosomes (Korban et al., 2009; Zonneveld, 2009, 2010; Höfer and Meister, 2010). It is recognized that the nuclear DNA content is a specific feature and its flow cytometric evaluation can be one of the methods helpful in differentiating taxa. For many apple genotypes, including all the Polish ones, these features are not known. In general, polyploidy is associated with enhanced nuclear DNA content that translates into larger nuclei, cells and stomata sizes. It is often related to variation in morphological, physiological or phenological characteristics such as, e.g., larger leaves, flowers and fruits or late flowering time (Webster, 1996; Jaskani and Khan, 2000; Ramanna and Jacobsen, 2003; Anssour et al., 2009; Rogalska et al., 2007; Podwyszyńska et al., 2015).

For the genotypes gathered in the gene bank of the Research Institute of Horticulture (InHort), DNA content evaluation and stomata measurement (easy to evaluate marker of increased ploidy level) will be very valuable for both scientific and practical purposes, with possible application in breeding aimed at creation of new cultivars with different morphological traits.

Nuclear DNA content was evaluated in relation to leaf, flower, fruits, stomata and pollen grain sizes as well as to the flowering time and pollen viability.

MATERIAL AND METHODS

PLANT MATERIAL

Seventy cultivars of cultivated apple (*Malus* × *domestica* Borkh.), including 46 Polish ones, derived from germplasm collection of the Research Institute of Horticulture were used for the study.

GENOME SIZE (NUCLEAR DNA CONTENT)

Analysis of genome size was done using flow cytometry (FCM/PI) (CyFlow PA, Partec, Germany). Samples were taken in mid-July from six leaves collected randomly from one plant of each analyzed cultivar. Leaf tissue (0.5–1 cm^2) was chopped together with a piece (1 cm^2) of plant internal standard in a Petri dish in 0.5 ml nuclei isolation Galbraith's buffer (Galbraith et al., 1983) to which propidium iodide (50 $mg \cdot ml^{-1}$) and RNase (50 $mg \cdot ml^{-1}$) were added (Śliwińska, 2008). As an internal standard, the young leaves of *Zea mays* CE-777 (2C=5.43 pg DNA) were used (Lysák and Doležel, 1998). The seed of *Zea mays* were kindly provided by the Institute of Experimental Botany, Olomouc, Czech Republic. After adding 1.5 ml of the isolation buffer, the samples were filtered through a 30 μm filter and incubated for 50–60 min at room temperature. The fluorescence of the nuclei was measured using a CyFlow Ploidy Analyser with CyView software (CyFlow PA, Partec, Germany) with an Nd-YAG green laser at 532 nm. The data were analysed by means of CyView software (Partec). The 2C DNA content of a sample was calculated as the sample peak mean divided by 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 in two runs from each nuclei isolation extract. The nuclear (2C) DNA content was evaluated for 70 genotypes.

PHENOTYPE EVALUATION

In each year of the study the dates of the beginning, full bloom and end of flowering were noted. For this study the Fleckinger scale (1948) was used. When the trees were in full bloom the flower size was evaluated by measuring the diameter of the 2nd and 3rd flower in the inflorescence (Anonymous, 2006). For each genotype flowers in 10 inflorescences were measured. For selected genotypes, representing all ploidy levels, the leaf size was evaluated. The leaf samples were collected in August when the vegetative growth of shoots was finished. The leaf area was measured for randomly collected 30 leaves, with an optical planimeter Delta–T Devices. The fruit weight was evaluated on apple samples, harvested in four replicates, each consisting of 15 pieces. The apples were weighed individually using a laboratory balance, with an accuracy of 1g.

Additionally, lamina thickness was measured with a caliper Sylvac System VIS for diploid cultivars 'Jonathan' and 'McIntosh' as well as their mixoploid (ploidy chimeras) counterparts possessing diploid and tetraploid genomes: 'Jonathan 2-4-4' with known ploidy levels of particular histogenic layers: diploid L1 and tetraploid L2 and L3 and 'McIntosh 2x + 4x' with unspecified ploidy level for individual histogenic layers. Five measurements were taken on the middle part between vascular bundles of each lamina of 50 leaves. Also, for these four cultivars, the relative chlorophyll content of leaves was performed by measuring the chlorophyll concentration index (CCI) as a ratio of optical transmission at 931 nm

divided by transmission at 653 nm using a MINOLTA Chlorophyll Meter SPAD. Five measurements were taken on the middle part between vascular bundles of each lamina of 50 leaves.

Phenotype and nuclear DNA content measurements were conducted from 2013 to 2014. Leaf measurements were taken in August and September. The values in Table 1 come from 2013 or 2014 (as described).

STOMATA LENGTH

The stomata were measured using light microscopy. The leaf samples 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 next mounted on slides for microscopic observations, according to the procedure of Dyki and Habdas (1996). The stomata measurements were determined for five/six leaves (x 10 stomata) of each genotype using a Nikon Eclipse 80i microscope with the program NIS-Elements BR 2.30, at 400 times magnification. The samples were collected from five diploid and seven triploid cultivars as well as from two mixoploid cultivars possessing tetraploid and diploid genomes ('Jonathan 2-4-4' and 'McIntosh 2x+4x').

POLLEN GRAIN LENGTH AND VIABILITY

A mixed sample of pollen from 5–10 anthers taken from six flowers of each analysed genotype was stained with acetocarmine. The measurements of pollen grain length were performed using a microscope as described for stomata measurements (see above). The pollen viability was checked with a fluorescence microscope (Nikon Eclipse 50i) with the program NIS-Elements BR 2.30.

The pollen germination was tested on microscope slides with different sucrose solutions (5, 10 and 20%) after 6 h of incubation at room temperature (Niles and Quesenberry, 1992). For each sucrose concentration, from 50 to 150 pollen grains were observed. Pollen germination was also evaluated on the stigma pistil in the process of self-pollination. The stigma pistils removed from flowers were stained with aniline blue after being macerated in 1% NaOH at 60°C for observation of viable, germinated pollen grains (Dyki, 1978).

The pollen was collected from five diploid and seven triploid cultivars and from mixoploid 'McIntosh 2x+4x'.

STATISTICAL ANALYSIS

All the parameters were analysed with ANOVA-nested design. All the calculations were done with the STATISTICA package (StatSoft v. 10). The means were compared by Tukey's test at $p=0.05$.

RESULTS

NUCLEAR DNA AMOUNT

For standard cultivars of the known chromosome number, 2C value was 1.71 pg for the diploid cultivar 'Alwa' (2n=2x=34) and 2.55 and 2.48 pg for triploid (3x=51) 'Boskoop' and 'Jonagold' (Tab. 1), respectively. In the case of 'McIntosh', which was previously regarded as a homogeneous tetraploid (4x=68), FCM analysis has shown that it has a mixoploid nature, possessing both diploid (1.72 pg) and tetraploid (3.37 pg) DNA amounts. In 61 cultivars (including 41 Polish ones), 2C DNA values ranged from 1.58 to 1.78 pg indicating their diploid chromosome number. Five Polish cultivars were identified as triploids ('Bursztówka Polska' 'Pagacz', 'Rapa Zielona', 'Rarytas Śląski' and 'Witos') owing to their nuclear DNA amounts ranging between 2.42 and 2.58 pg. Standard deviation (SD) for measurements of nuclear DNA contents ranged between ±0.01 and ±0.07.

PHENOTYPE

The leaf, flower, fruit, stomata and pollen grain sizes were on average significantly larger in triploids (Tab. 1, 2). In diploids the leaf area was on average 36.0 cm^2, whereas in triploid cultivars – 49.1 cm^2. Within diploids, the smallest leaves were noted for 'Redkroft', 'Koral' and 'Bukówka' (ca. 20 cm^2) and the largest ones for 'Sebastian' and 'Cortland Wicki' (ca. 54 and 57 cm^2). In triploids the leaf size ranged from 32.9 in 'Witos' to 70.7 cm^2 in 'Rapa Zielona'. In mixoploid 'McIntosh', possessing both diploid and tetraploid genomes, the leaf area was much bigger than in diploid 'McIntosh', 64.2 and 41.8 cm^2, respectively (Tab. 3). Moreover, the leaves of the ploidy chimeras 'McIntosh 2x+4x' and 'Jonathan 2-4-4' were wider and more rounded than in the diploids.

The leaf thickness was significantly higher in mixoploids 'Jonatan 2-4-4' and 'McIntosh 2x+4x', compared to their diploid counterparts (Tab. 3). In 'Jonathan 2-4-4', the chlorophyll content was also higher than in diploid 'Jonathan', whereas in 'McIntosh' this trait was similar in mixoploid and diploid (Tab. 3).

The flower diameter of diploids was on average 46.1 mm and triploids 52.4 mm (Tab. 1, 2). The smallest flower diameter in diploids of ca. 38–39 mm was found in 'Piękna z Rept' and 'Perła', the biggest one of 60.7 mm in 'Profesor Jankowski'. In triploids, the flower diameter ranged from 42.5 mm in 'Pagacz' to 58.7 mm in 'Boskoop'. Interestingly, in the cultivar 'McIntosh 2x+4x' possessing both diploid and tetraploid genomes, the flower diameter was 47.4 mm and did not differ significantly from that of diploid 'McIntosh' (44.5 mm).

TABLE 1. Nuclear (2C) DNA content, flower, leaf and fruit sizes of 70 cultivated apple cultivars including Polish ones

Cultivar	Nuclear (2C) DNA content (pg) 2013	Ploidy level	Flower diameter (mm) 2013	Leaf area (cm^2) 2014	Fruit weight (g) 2014
'Ligostar'*	1.58±0.04 a	2x	42.7±2.02 a-h	40.0±10.65 e-k	257.5± 9.4 z
'Szampion'	1.58±0.04 a	2x	–	–	157.0±13.1 e-r
'Bastek'*	1.63±0.06 a-c	2x	44.8±4.39 c-j	30.0±7.15 b-g	128.5±6.6 a-g
'Jonathan'	1.63±0.05 a-c	2x	45.3±2.31 a-g	26.2±7.04	126.8±12.3 a-g
'Golden Delicious'	1.64±0.06 a-d	2x	–	–	156.8±13.4 d-p
'Medea'*	1.64±0.06 a-e	2x	46.7±2.10 f-k	25.9±5.89 a-c	146.0±9.6 c-m
'Odra'*	1.64±0.03 a-e	2x	47.1±2.88 f-l	51.4±10.04 n-r	138.0±10.0 c-i
'Priam'	1.65±0.03 a-f	2x	–	–	93.3±3.0 ab
'U 1165'*	1.65±0.04 a-f	2x	47.1±3.63 g-l	35.0±8.19c-j	175.0±17.3 i-t
'Melfree'*	1.66±0.05 b-g	2x	50.9±2.60 k-o	33.4±6.28 c-i	178.0±6.1 j-u
'Koral'*	1.66±0.01 b-g	2x	47.6±5.1 h-l	21.0±6.89 ab	190.5±23.7 o-v
'Gala'	1.66±0.03 b-g	2x	–	–	143.5±6.2 c-l
'Marwit'*	1.66±0.05 c-g	2x	51.9±1.79 l-p	35.2±7.79 c-j	155.5±1.3 d-p
'Free Redstar'*	1.66±0.03 c-h	2x	49.0±1.51 i-m	31.6±4.27 c-h	176.0±5.7 j-t
'Egeria'*	1.67±0.01 c-i	2x	44.9±2.00 d-k	44.7±11.97 j-p	146.8±17.3 c-n
'Bancroft'	1.67±0.04 c-i	2x	–		125.8±9.2 a-f
'Fantazja'*	1.67±0.02 c-j	2x	46.4±4.10 d-k	28.2±4.85 a-f	146.8±2.6 c-n
'Primula'*	1.68±0.06 c-k	2x	47.1±3.20 g-l	29.9±6.96 b-g	144.3±10.2 c-l
'Ligolina'*	1.68±0.02 c-k	2x	46.9±1.79 f-l	40.8±10.88 f-m	187.5±2.6 m-w
'Lodel'*	1.68±0.07 c-k	2x	51.0±1.70 k-o	27.3±4.05 a-d	150.0±14.9 c-o
'Idared'	1.68±0.03 c-k	2x	–	–	164.0±20.3 e-r
'Freedom'	1.68±0.05 c-l	2x	–	–	147.8±8.4 c-n
'Idaredest'*	1.68±0.03 c-l	2x	41.3±2.35 a-d	–	220.0±11.4 w-z
'Najdared'*	1.69±0.03 c-m	2x	40.0±1.63 a-c	–	188.3±4.3 n-v
'Ligol'*	1.69±0.03 c-m	2x	50.8±2.66 k-n	37.3±9.32 d-k	235.5±27.1 x-z
'Red Delicious'	1.69±0.04 c-n	2x	–	–	213.3±20.4 s-y
'Redspur Delicious'	1.69±0.04 c-n	2x	–	–	–
'Delikates'*	1.69±0.04 c-n	2x	48.5±3.24 i-m	36.3±7.60 c-j	–
'Szampion Reno'*	1.69±0.03 c-n	2x	41.4±0.97 a-d	28.9±5.36 a-g	150.0±2.2 c-o
'Perła'*	1.69±0.04 c-n	2x	38.6±1.96 a	27.1±7.74 a-d	144.8±3.6 c-l
'Sebastian'*	1.70±0.02 c-o	2x	46.1±3.00 e-k	54.2±10.55 pr	140.5±15.1 c-k
'Cortland'	1.70±0.02 c-p	2x	–	–	219±.05.5 u-z
'Piękna z Rept'*	1.70±0.03 c-p	2x	37.8±2.53 a	45.6±11.73 k-p	116.0±2.2 a-d
'Redkroft'*	1.71±0.03 c-r	2x	47.3±3.80 h-l	19.1±4.94 a	193.8±8.7 p-x
'Alwa'*	1.71±0.06 d-r	2x	48.5±3.14 i-m	41.4±7.00 h-n	161.8±12.0 e-r
'Rajsmak'*	1.71±0.04 d-r	2x	44.6±3.20 b-h	38.5±9.50 g-l	149.8±14.1 c-o
'Mnichy Polskie'*	1.71±0.04 d-r	2x	44.9±4.40 c-j	52.3±9.21 o-r	128.0±5.9 a-g

Cultivar	Nuclear (2C) DNA content (pg) 2013	Ploidy level	Flower diameter (mm) 2013	Leaf area (cm^2) 2014	Fruit weight (g) 2014
'Delcorf'	1.71±0.02 d-r	2x	–		159.8±21.2 e-r
'Kosztela'*	1.71±0.04 e-r	2x	56.0±2.98 o-s	30.4±5.32 b-g	167.5±30.7 f-r
'Linda'	1.71±0.02 e-r	2x	–		168.5±4.5 g-r
'Chopin'*	1.71±0.03 e-r	2x	41.5±2.55 a-e	38.1±8.10 f-k	178.5±17.5 j-w
'McIntosh'	1.72±0.04 e-r	2x	44.5±3.95 c-i	41.8±8.04 h-n	187.0±10.4 m-w
'Cortland Wicki*	1.72±0.04 f-r	2x	49.5±3.21 j-n	57.4±11.03 r	244.5±37.8 y-z
'Sawa'*	1.72±0.04 f-r	2x	49.5±2.95 j-n	37.6±6.04 e-k	174.3±17.7 i-s
'Profesor Jankowski'*	1.72±0.02 f-r	2x	60.7±1.50 s	42.3±9.74 i-o	134.0±7.7 b-i
'Golden Dream'*	1.72±0.01 f-r	2x	39.6±1.07 a-b	40.8±12.60 h-m	211.8±6.7 s-y
'Reneta Kurska'	1.72±0.04 f-r	2x	–	–	146.3±20.6 c-m
'Melrose'	1.73±0.02 h-r	2x	–	–	129.0±20.4 a-g
'Braeburn Arno'*	1.74±0.01 h-r	2x	41.1±1.91 a-c	31.8±6.30 c-h	159.5±7.0 e-r
'Czerwony Lampart'*	1.74±0.04 h-r	2x	41.8±2.10 a-f	37.2±6.27 d-k	185.0±9.0 l-w
'Worcester Pearmain'	1.74±0.01 h-r	2x	–	–	1. –
'Lobo'	1.74±0.05 i-r	2x	–	–	159.3±14.4 e-r
'Juga'	1.75±0.04 j-r	2x	50.8±2.70 k-n	29.3±7.54 b-g	212.0±6.0 s-y
'James Grieve Lired'	1.75±0.03 k-r	2x	–	–	154.0±12.1 c-p
'Bukówka'*	1.76±0.03 l-r	2x	50.4±2.55 k-n	21.2±4.85 ab	87.3±3.0 a
'Waleria'*	1.76±0.01 m-r	2x	40.9±2.70 a-c	28.0±6.08 a-e	180.8±4.3 k-w
'Gold Milenium'*	1.76±0.03 n-r	2x	48.2±3.29 i-m	33.1±8.93 c-i	124.8±7.1 a-e
'Goldeniszyk'*	1.77±0.03 o-r	2x	50.4±2.00 k-n	29.5±8.43 b-g	112.3±2.2 a-c
'Warta'*	1.77±0.03 o-r	2x	42.1±3.21 a-g	33.8±9.58 c-i	142.8±7.1 k-w
'Gala Natali'*	1.78±0.04 r	2x	40.8±1.14 a-c	48.2±13.81 l-r	151.8±2.5 c-o
'Delela'*	1.78±0.03 r	2x	40.8±1.40 a-c	50.9±40.81 n-r	171.8±8.7 h-s
'Witos'*	2.42±0.03 st	3x	52.9±1.85 m-p	32.9±8.13 c-h	216.3±10.9 t-z
'Rarytas Śląski'*	2.47±0.02 st	3x	49.6±2.88 j-n	38.1±7.06 f-k	162.8±3.6 e-r
'Rapa Zielona'*	2.47±0.05 st	3x	56.2±3.74 p-s	70.7±13.53 s	197.5±49.9 r-x
'Jonagold'	2.48±0.05 s-w	3x	54.2±3.56 n-r	45.6±8.40 k-p	229.5±7.3 v-z
'Bursztówka Polska'*	2.51±0.05 t-v	3x	53.2±3.36 m-p	53.3±12.34 p-r	130.0±8.0 b-h
'Boskoop'	2.55±0.04 uv	3x	58.7±3.95 rs	50.5±11.44 m-r	211.8±5.4 s-y
'Pagacz'*	2.58±0.04 wv	3x	42.5±2.27 a-h	53.0±10.68 p-r	239.5±16.4 y-z
'Red Jonaprice'	2.60±0.05 v	3x	53.0±2.92 m-p	48.5±9.05 m-r	242.0±17.3 y-z
'McIntosh' (4x)	1.72±0.02 e-r 3.37±0.03 x	4x+2x	47.4±2.76 m-p	64.2±8.04 i-o	205.0±5.4 s-x

Means ± SD in columns followed by the same letters do not differ significantly at significance level p=0.05, Tukey's test;
* 46 cultivar of Polish origin.

No significant differences in the flowering time between diploid and triploid cultivars were found (Tab. 2). Cultivars of both ploidy levels flowered on average on 8 May (2014).

The fruit weight of diploids was on average 162.7 g and in triploids it was significantly higher (204.7 g) (Tab. 2). The lowest fruit weight in diploids of approximately 90 g was found in 'Bukówka' and 'Priam' and the highest one of 257.5 g in 'Ligostar'; besides, 7 other diploid cultivars had fruits weighing more than 200 g ('Golden Dream', 'Juga', 'Red Delicious', 'Cortland', 'Idarest', 'Ligol' and 'Cortland' Wicki') (Tab. 1). In triploids, the fruit weight ranged from 130 g in 'Bursztówka Polska' to ca. 240 g in 'Red Jonaprice' and 'Pagacz', the mutants of 'Jonagold' with fruit weight of ca. 230 g; and no significant differences were recorded between 'Jonagold' and its mutants.

In the triploid cultivars, except for 'Rapa Zielona' (27 μm), the stomata were significantly larger than those of the diploid cultivars (Tab. 2, Fig. 1a–f, 2a). Stomata length was on average 28.4 μm in diploids and 32.1 μm in triploids. No differences in the stomata size between diploids and mixoploids either of 'Jonathan 2-4-4' or 'McIntosh (2x+4x)' were observed (Tab. 3).

The pollen grain diameter, on average, differed significantly between diploids (30.7 μm) and triploids (33.7 μm) with the largest grains (ca. 35 μm) observed in triploids, 'Pagacz' and 'Witos' as well as diploid 'Ligolina' (Tab. 2, Fig. 2b, 3a,b). The smallest (on average) pollen grains were found in diploid and mixoploid 'McIntosh' (26.4 and 29.7 μm, respectively); besides, the highest variation in the grain size was observed in the mixoploid, from very small (13.6 μm) to very large grains (57.1 μm) (Tab. 3, Fig. 2b, Fig. 3c,d,e).

The pollen grain viability evaluated in 20% sucrose solution was generally distinctly higher in diploids compared to triploids (on average 75.6% and 22.8, respectively) (Tab. 2, 4). However, large differences in pollen viability were noted depending on sucrose concentration (Tab. 4, Fig. 3f–i). In general, the pollen grains of the diploid cultivars germinated in higher percentage in 20% sucrose solution, while triploid cultivars at 5% sucrose. The biggest differences in pollen germination were noted for triploid 'Rapa Zielona', which germinated in 65% at 5% sucrose, while only in 8.1% at 10% sucrose. Mixoploid 'McIntosh' had one of the lowest pollen germination potential (on average 14%). Generally, the frequency of pollen germination on stigma, tended to be comparable to germination in sucrose solutions (Fig. 3j–m).

DISCUSSION

Our study indicated that 10.9% of the evaluated Polish cultivars were triploids, just as it has been estimated for the general distribution of triploids in the entire pool of cultivars (Janick, 1996). Pereira-Lorenzo et al. (2007) informed that among 140 apple cultivars, including 114 local Spanish genotypes, 39 were identified as triploids (ca. 28%). Janick (1996) reported that the frequency of triploidy in a population from diploid parents is at the rate of 0.3%. Recently, Considine et al. (2012) proved that

TABLE 2. Comparison of nuclear DNA content (2C) and several phenotype traits of diploid and triploid apple cultivars

Trait	Ploidy level				p
	2x		3x		
	Mean	Min-Max	Mean	Min-Max	
Flowering time	8–9 May	7–13 May	8 May	7–11 May	0.29
Flower diameter (mm)	46.1 a	37.8–60.7	52.4 b	42.5–58.7	0.00
Fruit weight (g)	162.7 a	87.3–257.5	204.7 b	130.0–242.0	0,00
Leaf area (cm^2)	36.0 a	19.1–57.4	49.1 b	32.9–70.7	0.00
Stomata length (μm)	28.4 a	26.3–29.7	32.1 b	27.9–35.1	0.00
Pollen grain diameter (μm)	30.7 a	26.4–35.7	33.7 b	31.37–35.3	0.05
Pollen grain germination (%)	75.6 b	48.6–86.1	22.8 a	4.0–49.5	0.00
Nuclear DNA content (pg)	1.70 a	1.58–1.78	2.51 b	2.42–2.60	0.00

Means in rows followed by the same letter do not differ at significance level p=0.05; Tukey's test.

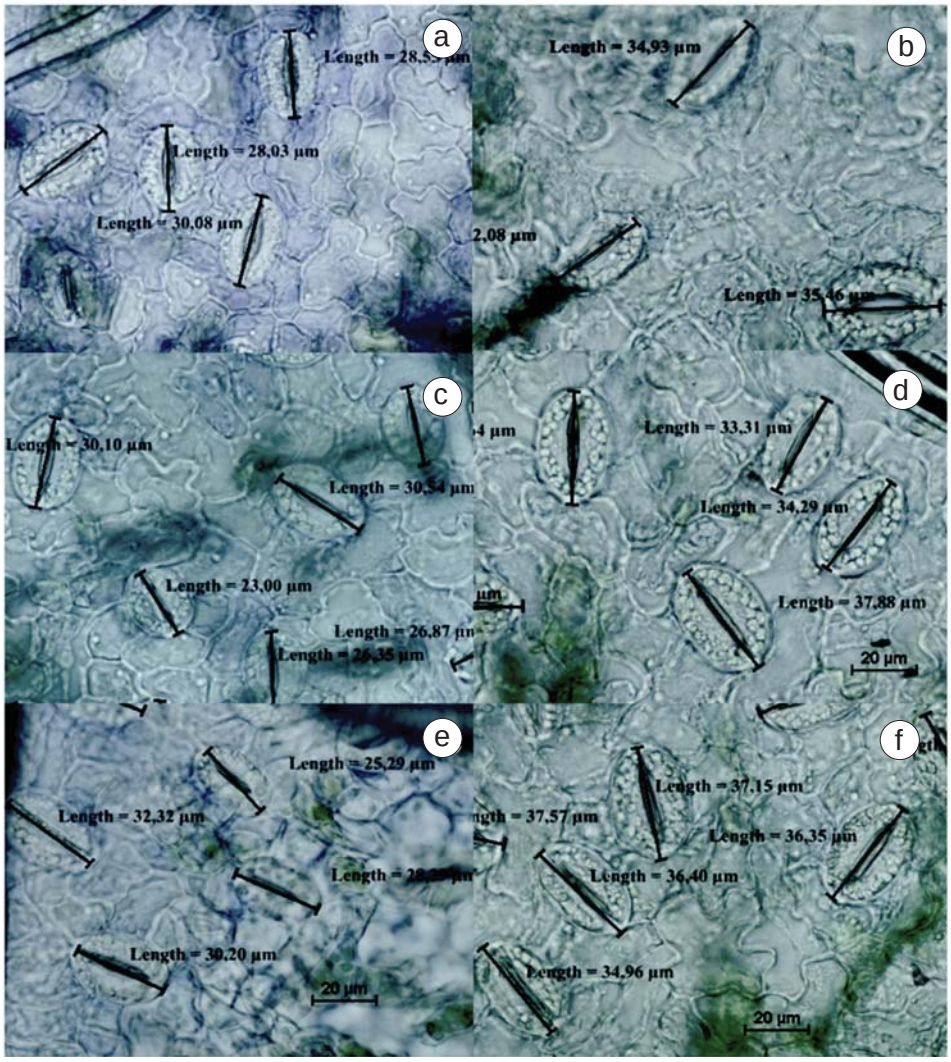

Fig. 1. Stomata of cultivated apple cultivars. (**a**, **c**, **e**) Diploids: 'Alwa', 'Ligol' and 'Ligolina', respectively. (**b**, **d**, **f**) Triploids: 'Boskoop', 'Bursztówka Polska' and 'Witos'. Bars: 20 µm.

in F_1 population derived from diploid parents there were only 0.199% triploids, 0.05% tetraploids and 0.778% aneuploids. In our research only one triploid 'Witos' cv. is a confirmed seedling of diploid parents, whereas 'Rarytas Śląski' is a seedling of triploid 'Blenheim Orange', and 'Pagacz' is a mutant of triploid 'Jonagold'. Other Polish triploid cultivars 'Rapa Zielona' and 'Bursztówka Polska' do not have a well documented origin.

Rybin (1926) (as cited in Crane and Lawrence, 1930) first observed triploids in *M.* × *domestica* based on chromosome counting. The ploidy level as well as DNA content of apple was first estimated using flow cytometry by Arumuganathan and Earle (1991). These authors assessed the nuclear DNA amount for three cultivars of *M.* × *domestica* (1.54–1.65 pg). Later, Dickson et al. (1992) evaluated 2C DNA of 25 apple species and cultivars and found that the diploid genome size showed little variation within the genus *Malus*. 2C values of 17 diploid *Malus* species from five sections ranged only from 1.21 to 1.67 pg and within *M.* × *domestica* from 1.50 to 1.73 pg with SD from 0.20 to 0.33. Besides, the 2C DNA content of 'Jonagold' (2.51 pg) evaluated by Dickson et al. (1992) was similar to our results (2.48 pg ±0.05) (Tab. 5). Höfer and Meister (2010) reported lower DNA values for eight genotypes of *M. domestica* with median of 1.51 pg compared to our results and those of Korban et al. (2009). Among 100 *Malus* accessions (species and hybrids including *M.* × *domestica*), the authors found 96 diploids (1.50–1.72 pg), three triploids (2.27–2.41 pg) and one tetraploid (3.13 pg). Comparison of the 2C values of apple reported in our and other stud-

TABLE 3. Comparison of several phenotype traits between diploid apple cultivars 'McIntosh' and 'Jonathan' and their ploidy chimeras possessing diploid and tetraploid genomes (2x+4x)

Trait	'McIntosh'		'Jonathan'	
	2x	2-4-4 or 2-4-2	2x	2-4-4
Leaf area (cm^2)	41.8 a	64.2 b	37.9 a	33.8 a
Lamina thickness (mm)	0.29 a	0.32 b	0.28 a	0.31 b
Chlorophyll concentration index	49.4 a	50.1 a	51.8 a	55.4 b
Stomata length (μm)	28.5 a	28.0 a	30.9 a	31.6 a
Pollen grain diameter (μm)	26.4 a	29.7 a	–	–
Pollen grain germination (%)	48.3 b	11.7 a	–	–
Pollen grain well formed (%)	57.5 b	36.4 a	–	–

Means in rows, separately for each cultivar followed by the same letter do not differ at significance level p=0.05; Tukey's test.

TABLE 4. Pollen germination of diploid and triploid cultivars in different sucrose solutions (5, 10 and 20%)

Cultivar	Pollen grain germination (%)			Average
	Sucrose concentration (%)			
	5	10	20	
Diploids				
'Alwa'	77.7	51.4	76.4	74.2
'Gold Milenium'	78.4	75.0	83.4	80.9
'Ligol'	70.5	15.2	86.1	56.7
'Ligolina'	50.0	–	83.4	82.1
'McIntosh'	–	–	48.6	48.6
Average	*69.2*	*47.2*	*75.6*	68.5
Triploids				
'Boskoop'	34.0	–	14.9	26.1
'Bursztówka Polska'	6.0	27.0	23.7	18.5
'Jonagold'	26.0	15.9	49.5	32.9
'Pagacz'	39.3	–	26.3	36.4
'Rapa Zielona'	65.0	8.1	27.4	33.1
'Red Jonaprince'	22.9	7.3	13.5	15.1
'Witos'	35.8	1.6	4.0	13.0
Average	*33.1*	*12.0*	*22.8*	25.0
Mixoploid				
'McIntosh' 2x+4x	19.4	9.8	11.7	14.0

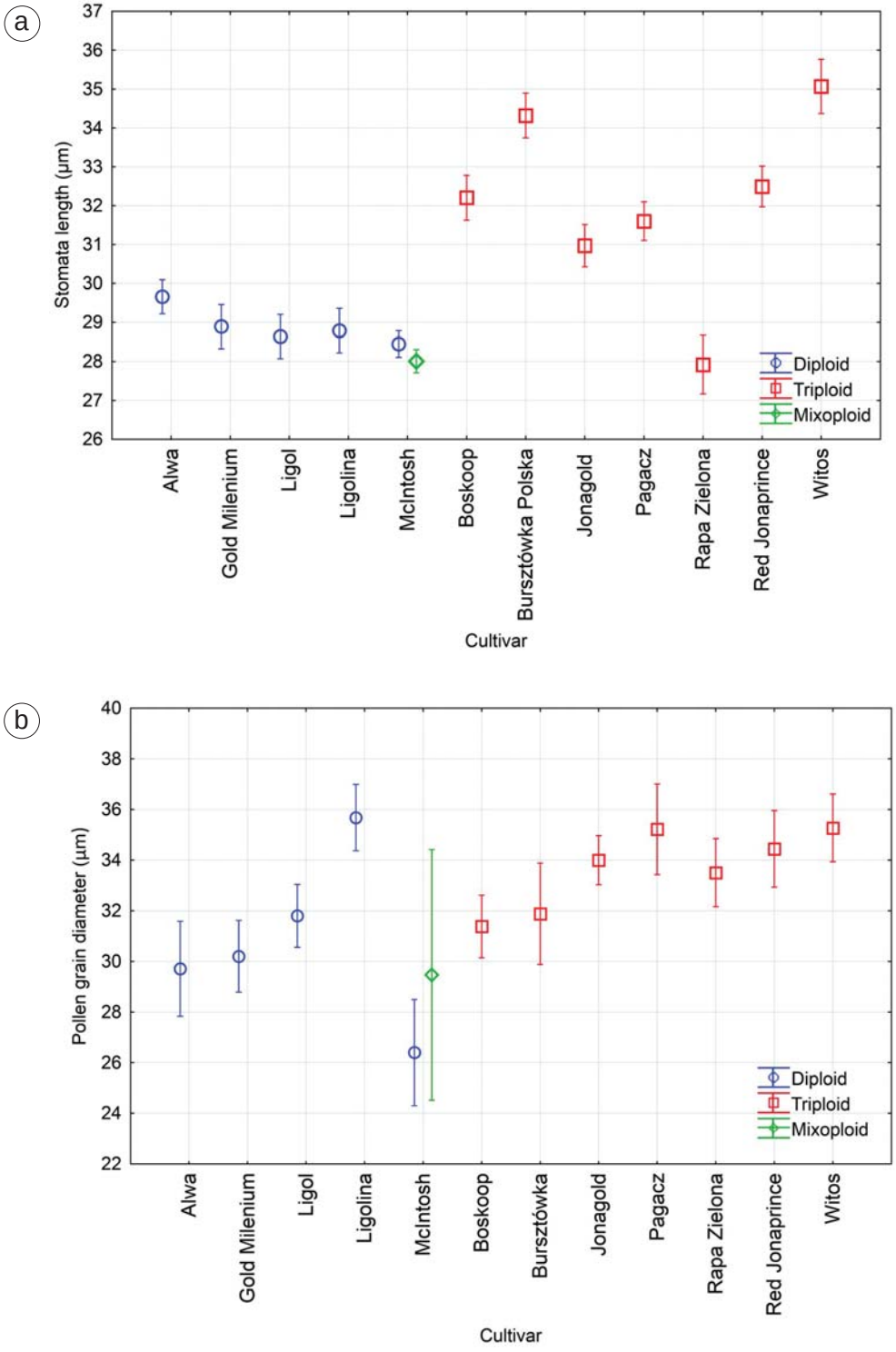

Fig 2. (**a**) Stomata length (mean ± SE, standard error) of cultivated apple cultivars of various ploidy level. (**b**) Pollen grain diameter (mean ± SE, standard error) of cultivated apple cultivars of various ploidy level. 'McIntosh' is represented by its two ploidy forms: (○) diploid and (◊) mixoploid (2x+4x).

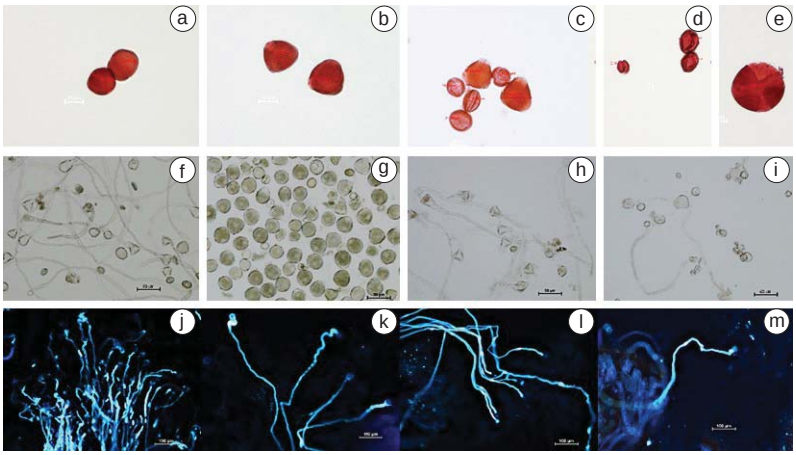

Fig. 3. Pollen grains of cultivated apple cultivars. (**a**, **f**, **j**) Diploid 'Gold Milenium'. (**b**, **g**, **k**) Triploid 'Witos'. Diploid 'McIntosh' (**c**, **h**, **l**). Mixoploid 'McIntosh' (**d**, **e**, **i**, **m**). Pollen grains of the particular cultivars: (**a**, **b**, **c**, **d**, **e**) acetocarmine stained, bars: 10 μm; (**f**, **g**, **h**, **i**) germinating in 20% sucrose solution, bars: 20 μm; (**j**, **k**, **l**, **m**) pollen grains germinating on stigma, bars: 100 μm.

ies shows that only two values (of 'Cortland' and 'Idared') exceed the margin of error (Tab. 5). The differences between our and other authors' estimations probably resulted from different FCM procedures (buffers, staining time and temperature, and internal standards) as well as various types of instruments. We obtained results more similar to those of Tatum et al. (2005) and Korban et al. (2009). Presumably, this is because they used the maize (line W22, 2C=5.35 pg) as internal standard and 1h staining time as we did. We used, however, another maize genotype (CE-777, 2C=5.43 pg). Instead, Höfer and Meister (2010) showed relatively lower nuclear DNA contents in apple since they used radish (2C=1.1 pg) as an internal standard and very short time of staining (1 min). Moreover, the range of standard errors (SDs) calculated for means of genome sizes in our study was comparable to those reported by Korban et al. (2009) and Tatum et al. (2005). All these data indicated a high degree of reliability of nuclear DNA evaluation in the current study.

Our results of phenotype evaluation confirmed that in apple, the higher ploidy level of triploids is generally associated with increased sizes of stomata, leaves, flowers and/or fruits as in many other plant genera such as tea (Wachira, 1994), banana, plantain (Vandenhout et al., 1995) and mulberry (Laltanmawii and Roychowdhuri, 2010). Pereira-Lorenzo et al. (2009) reported that apple triploids produce on average 15% heavier apples, whereas we found that the fruits of triploids were on average 25.8% larger than those of diploids. We did not find, however, any clear correlation between the ploidy level and flowering time which was highly differentiated, irrespective of the ploidy level.

Our study showed that generally triploid cultivars had larger stomata (by 13%) than the diploid ones with only one exception of triploid 'Rapa Zielona' whose stomata had the same size as diploids. Similarly, high positive correlation between nuclear DNA amount and stomatal length of apple cultivars was observed by Tatum et al. (2005) and

TABLE 5. Comparison of nuclear DNA contents (2C) (means ± SD) of *Malus* × *domestica* cultivars analyzed in the present study and by other authors: Dickson et al., 1992 (D), Tatum et al., 2005 (T) and Korban et al., 2009 (K)

Cultivar	Estimation of the present study	Estimations of other authors
'Cortland'	1.70±0.02	1.57±0.06^{T}
'Gala'	1.66±0.03	1.57±0.14^{T}
'Golden Delicious'	1.64±0.06	1.62±0.02^{T}
'Idared'	1.68±0.03	1.59±0.02^{T}
'Redspur Delicious'	1.69±0.04	1.66±0.01^{K}
'Jonagold'	2.48±0.05	2.48±0.02^{T}. 2.51±0.2^{D}

Korban et al. (2009). The authors also reported that the ploidy chimera 'Kimball McIntosh 2-4-4-4' had the largest stomata. That is inconsistent with our observations. Thus, the mixoploid 'McIntosh', which has diploid and tetraploid cells (confirmed by cytometric analysis), had stomata length comparable to its diploid counterpart. A similar situation was observed for another ploidy chimera, cultivar 'Jonathan 2-4-4' which had stomata of the same size as diploid 'Jonathan'. It is widely accepted that dicotyledones are generally built of three apical histogenic layers so-called L1, L2 and L3. The L1 creates the epidermis with stomata, L2 forms the subepidermal tissue, e.g. mesophyll, together they are named tunica, and L3 forms the corpus with vascular tissue (Van Harten, 2002; Zonneveld, 2007). Gametes always originate in the L2 layer and roots in L3. In the light of such basic concept of a layered plant structure, our data indicate that L1 of ploidy chimera 'McIntosh' is diploid because of small stomata characteristic of diploid 'McIntosh'. This supposition can be further supported by the low ratio of diploid to tetraploid nuclei (ca. 0.5) estimated with FCM for chimera 'McIntosh'. Furthermore, some other traits such as leaf area and lamina thickness were significantly larger in this chimera, compared to its diploid counterpart. In turn, 'Jonathan 2-4-4' had significantly thicker lamina and a higher chlorophyll content than diploid 'Jonathan'. These phenotype characteristics of chimeric cultivars indicate their tetraploidy in L2 since tetraploids of apple (Lespinasse and Noiton, 1986; Blanke et al., 1994; Sedysheva and Gorbacheva, 2013) and many other species (Jaskani et al., 2005; Elradi and Unal, 2010; González-Rodríguez and Grajal-Martín, 2013; Podwyszyńska et al., 2015) have larger and thicker leaves and often a higher chlorophyll content. Our supposition corresponds well with the reports on *Hemerocallis* ploidy chimeras 2-4-4 and 2-4-2 whose stomata were the same size as in diploids but the leaf sizes were as in tetraploids (Arisumi, 1964; Podwyszyńska et al., 2011). We also found that chimera 'McIntosh 2x+4x' produced low viability pollen with large percentage of degenerated pollen grains. Since apple tetraploids are often characterized by low fertility (Sedysheva and Gorbacheva, 2013) and the fact that gametes arise from L2, the low pollen viability of chimera 'McIntosh' can further confirm that its L2 is tetraploid. The lower pollen viability of autotetraploid *Malus* × *domestica* plants has been attributed to instability of chromosome number during abnormal meiosis (Sedysheva and Gorbacheva, 2013). In conclusion, we suppose that mixoploid 'McIntosh' is probably a chimera type 2-4-4 or 2-4-2.

Our results concerning tetraploid apple chimeras correspond well with reports on tetraploidy in apple associated with larger, wider and less pointed leaves, larger stomata and lower pollen viability (Blanke et al., 1994; Sedysheva and Gorbacheva, 2013). Furthermore, the latter observed that the frequency of microsporogenesis disorders in tetraploid apple forms (11.7–61.1%) was much higher than in diploid apple genotypes 0.9–4.7% (Krylova, 1981; as cited in Sedysheva and Gorbacheva, 2013). These disorders in new tetraploid apple genotypes were related to abnormal divisions occurring in all consecutive stages of meiosis with the presence of micronuclei as a predominant type in telophase-I and telophase-II (Sedysheva and Gorbacheva, 2013). In the tetrad phase, as a consequence of the previous disorder, the presence of polyads was observed instead of normal tetrads as well as the presence of microspores with micronuclei. These findings can explain the high frequency of degenerated pollen grains and high variation in their size (from very small to large grains) found in 'McIntosh 2x+4x' during our studies.

We observed that all of the studied triploids produced some viable pollen. However, the average percentage of germinating pollen grains of triploids (25%) was much lower compared to that of diploids (68.5%). That is consistent with data reported as early as in 1930 by Crane and Lawrence who found that the percentage of pollen germination in triploid apple cultivars ranged from 4 to 27%, while in diploid cultivars from 50 to 97%. These authors further reported that odd multiple polyploids (including triploids) of *Rubus* and *Prunus* were relatively infertile, because seed development and fruit development in these genera are closely related and triploids are relatively unproductive. Instead, triploid genotypes of apple are productive. The apple has ten embryos and often a single seed is sufficient for the development of a fruit (Crane and Lawrence, 1930). This indicated parthenocarpy and renders fruit production still less dependent on formation of seeds. Fruit set in many apomictic apple genotypes may therefore be maintained in spite of a high degree of generational sterility. In turn, Bisognin et al. (2009) found that fruit set and seed formation were poor if a non-apomictic genotype was pollinated with pollen of an apomict triploid. These authors suggest that it was caused by low pollen fertility of triploids, since Schmidt (1964) reported that mature anthers of triploids contained 90% of degenerated pollen probably due to the asymmetry in the reduction division of meiosis.

In conclusion, despite the relative low pollen viability observed in triploids and chimera 'McIntosh 2x+4x', the studied plolyploid cultivars formed a considerable quantity of normal pollen with probable some diploid pollen, as proved by Sedysheva and Gorbacheva (2013). That means that either triploid or tetraploid genotypes can be used as pollinators in order to obtain new trip-

loid cultivars, which have been shown to possess new high-quality adaptive features (Janick, 1996; Pereira-Lorenzo et al., 2007; Sedysheva and Gorbacheva, 2013).

CONCLUSIONS

1. Significant differences in nuclear DNA amount were identified among either the three ploidy levels or within the cultivar groups representing the same ploidy level, diploids or tetraploids. 2C DNA values for diploids ranged from 1.58 to 1,78 pg, for triploids from 2.42 to 2.58 pg and 2C in tetraploid genotype was 3.37 pg.
2. Among commercially grown apple cultivars, the higher ploidy level is generally associated with increased sizes of flowers, leaves and fruits as well as pollen grains and stomata, but decreased pollen viability.
3. Stomata size is closely related to ploidy level and can be used as additional morphological marker discriminating diploids and triploids of *Malus* × *domestica*.
4. A correlation between ploidy level and flowering time was not confirmed.
5. In the case of mixoploid apple genotypes which possess diploid and tetraploid genomes, some phenotype observations are helpful in describing the ploidy levels of L1 and L2. Small stomata sizes (similar to diploid) indicate diploid L1 and larger leaf sizes compared to diploid counterparts show tetraploid L2.
6. The results will be useful for breeding, in which it is important to determine maternal and paternal genotypes as well as the direction of the crossing which is of great significance in obtaining seeds and materials for further selections.

AUTHORS' CONTRIBUTIONS

MP and DK – idea and study design; MP – flow cytometry analysis, statistical analysis and drafting of manuscript; DK and IS – phenotype evaluation; AM and BD – microscopic observations. The authors declare that there are no conflicts of interest.

ACKNOWLEDGEMENTS

This work was supported by the Polish Ministry of Science and Higher Education through statutory funds of the Research Institute of Horticulture, Skierniewice, Poland.

REFERENCES

Anonymous 2006. Protocol for distinctness, uniformity and stability tests, *Malus domestica* Borkh., APPLE (CPVO-TP/14/2, UPOV Species Code: MALUS_DOM, adopted on 14/03/2006).

Anssour S, Krugel T, Sharbel TF, Saluz HP, Bonaventure G, and Baldwin IT. 2009. Phenotypic, genetic, and genomic consequences of natural and synthetic polyploidization of Nicotiana attenuata and Nicotiana obtusifolia. *Annals of Botany* 103: 1207–1217.

Arisumi T. 1964. Colchicine-induced tetraploid and cytochimeral daylilies. *Journal of Heredity* 55: 255–261.

Arumuganathan K, and Earle ED. 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9: 208–218.

Bisognin C, Seemüller E, Citterio S, Velasco R, Grando MS, and Jarausch W. 2009. Use of SSR markers to assess sexual vs. apomictic origin and ploidy level of breeding progeny derived from crosses of apple proliferation-resistant *Malus sieboldii* and its hybrids with *Malus* × *domestica* cultivars. *Plant Breeding*: 507–513.

Blanke MM, Höfer M, and Pring RJ. 1994. Stomata and structure of tetraploid apple leaves cultured *in vitro*. *Annals of Botany* 73: 651–654.

Considine MJ, Wan Y, D'Antuono MF, Zhou Q, Han M, Gao H, and Wang M. 2012. Molecular genetic features of polyploidization and aneuploidization reveal unique patterns for genome duplication in diploid *Malus*. *PloS One* 7: e29449.

Crane MB, and Lawrence WJC. 1930. Fertility and vigour of apples in relation to chromosome number. *Journal of Genetics* 22: 153–163.

Dickson EE, Arumuganathan K, Kresovich S, and Doyle JJ. 1992. Nuclear DNA content variation within the Rosaceae. *American Journal of Botany*: 1081–1086.

Dyki B, and Habdas H. 1996. Metoda izolowania epidermy liści pomidora i ogórka dla mikroskopowej oceny rozwoju grzybów patogenicznych. (The method of isolation of epidermis of tomato and cucumber leaves for microscopic investigation of pathogenic fungus development). *Acta Agrobotanica* 49: 123–129 (in Polish with English abstract).

Dyki B. 1978. The use of a fluorescence technique for the investigation of self-sterility in breeding lines of cauliflower. *Biuletyn Warzywniczy* 21: 267–272 (In Polish with English abstract).

Dzialuk A, Chybicki I, Wele M, Śliwińska E, and Burszyk J. 2007. Presence of triploids among oak species. *Annals of Botany* 99: 959–964.

Elradi T, and Unal M. 2010. Production of colchicine-induced tetraploids in *Vicia villosa* Roth. *Caryologia* 63: 292–303.

Fleckinger J. 1948. Les studies végétatifs des arbres fruitiers en rapport avec les traitements. *Pomologie Française* (Suppl.): 81–93.

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, and Firoozabady E. 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science* 220: 1049–1051.

González-Rodríguez AM, and Grajal-Martín MJ. 2013. Physiological behaviour of mangos with different ploidy levels. *Acta Horticulturae* 992: 155–158.

Höfer M, and Meister A. 2010. Genome size variation in *Malus* species. *Journal of Botany* 2010: 1–9.

Janick J, Cummins JN, Brown SK, and Hemmat M. 1996. Apples. In: Janick J, and Moore JN (eds.). *Fruit Breeding: Tree and Tropical Fruits* 1, 1–77. John Willey and Sons, NY, USA.

Jaskani MJ, and Khan IA. 2000. Characterisation of interploid hybrids of Kinnow mandarin. Proceedings of International Society of Citri*culture* 1: 165–166.

Jaskani MJ, Kwon SW, and Kim DH. 2005. Comparative study on vegetative, reproductive, and qualitative traits of seven diploid and tetraploid watermelon lines. *Euphytica* 145: 259–268.

Jędrzejczyk I, and Śliwińska E. 2010. Leaves and seeds as materials for flow cytometric estimation of the genome size of 11 Rosaceae woody species containing DNA-staining inhibitors. *Journal of Botany*: 1–9.

Korban SS, Wannarat W, Rayburn CM, Tatum TC, and Raybur AL. 2009. Genome size and nucleotypic variation in *Malus* germplasm. *Genome* 52: 149–155.

Krylova VV. 1981. *Apple Embryology*. Kishinev, Shtiintza, 148 p.

Laltanmawii, and Roychowdhuri S. 2010. Effects of chromosomal variations on morphology and leaf anatomical behaviours in mulberry (*Morus* sp.). *Journal of Crop and Weed* 6: 35–39.

Lespinasse Y, and Noiton D. 1986. Contribution to the study of a haploid apple (*Malus pumila*). Descriptive study and comparison with clones of different levels of ploidy. 1. Vegetative characters: internodes, leaves and stomata. *Agronomie* 6: 659–664.

Lysák MA, and Doležel J. 1998. Estimation of nuclear DNA content in *Sesleria* (Poaceae). *Caryologia* 51: 123–132.

Niles WL, and Quesenberry KH. 1992. Pollen germination of rhizoma peanut cv. Florigraze. *Peanut Science* 19: 105–107.

Pereira-Lorenzo S, Ramos-Cabrer AM, and Díaz-Hernández MB. 2007. Evaluation of genetic identity and variation of local apple cultivars (*Malus* × *domestica* Borkh.) from Spain using microsatellite markers. *Genetic Resources and Crop Evolution* 54: 405–420.

Pereira-Lorenzo S, Ramos-Cabrer AM, and Fischer M. 2009. Breeding apple (*Malus* × *domestica* Borkh). In: Jain SM, and Priyadarshan PM (eds.), *Breeding Plantation Tree Crops: Temperate Species*, 33–81. Springer, New York.

Podwyszyńska M, Gabryszewska E, Jasiński A, and Sochacki D. 2011. Histogenic identification by cytological analysis of colchicine-induced polyploids of *Hemerocallis*. *Acta Horticulturae* 886: 245–250.

Podwyszyńska M, Gabryszewska E, Dyki B, Stępowska AA, Kowalski A, and Jasiński A. 2015. Phenotypic and genome size changes (variation) in synthetic tetraploids of daylily (*Hemerocallis*) in relation to their diploid counterparts. *Euphytica* 203: 1–16.

Ramanna MS, and Jacobsen E. 2003. Relevance of sexual polyploidization for crop improvement – a review. *Euphytica* 133: 3–8.

Ramos-Cabrer AM, Diaz-Hernandez MB, and Pereira-Lorenzo S. 2007. Morphology and microsatellites in Spanish apple collections. *Journal of Horticultural Science and Biotechnology* 82: 257–265.

Rogalska SM, Achrem M, and Kalinka A. 2007. Mechanizmy zmian genomowych i zmian w ekspresji genów w mieszańcowych poliploidach roślin. *Kosmos* 56 (3–4): 421–433.

Rybin VA. 1926. Cytological investigations of the genus *Malus* (preliminary account). *Bulletin of Applied Botany and Plant Breeding* (Leningrad) 16: 187–200.

Schmidt H. 1964 Beiträge zur Züchtung apomiktischer Apfelunterlagen. *Zeitschrift für Pflanzenzüchtung* 52: 27–102.

Sedysheva GA, and Gorbacheva NG. 2013. Estimation of new tetraploid apple forms as donors of diploid gametes for selection on a polyploidy level. *Universal Journal of Plant Science* 1(2): 49–54.

Śliwińska E. 2008. Zastosowanie cytometrii przepływowej do oznaczania zawartości DNA u roślin (Estimation of DNA content in plants using flow cytometry). *Postępy Biologii Komórki* 35, Suppl 24: 165–176 (in Polish with English abstract).

Tatum TC, Stepanovic SS, Biradar DP, Rayburn AL, and Korban SS. 2005. Variation in nuclear DNA kontent in *Malus* species and cultivated apples. *Genome* 48: 924–930.

Vandenhout H, Ortiz R, Vuylsteke D, Swennen R, and Bai KV. 1995. Effect of ploidy on stomatal and other quantitative traits in plantain and banana hybrids. *Euphytica* 83: 117–122.

Van Harten AM. 2002. Mutation breeding of vegetatively propagated ornamentals. In: Vainstein A. (ed.). *Breeding for Ornamentals: Classical and Molecular Approaches*, 105-127. Kluwer Academic Publishers, Netherlands.

Wachira FN.1994. Triploidy in tea (*C. sinensis*): effects of yield and yield attributes. *Journal of Horticultural Science* 69: 53–60.

Webster AD. 1996. Cherry rootstock evaluation at East Malling. *Acta Horticulturae* 410: 247–255.

Zonneveld BJM. 2007. Nuclear DNA content of ploidy chimeras of *Hosta* Tratt. (Hostaceae) demonstrate three apical layers in all organs, but not in the adventitious root. *Plant Systematics and Evolution* 269: 29–38.

Zonneveld BJM. 2009. The systematic value of nuclear genome size for "all" species of *Tulipa* L. (Liliaceae). *Plant Systematics and Evolution* 281: 217–245.

Zonneveld BJM. 2010. The involvement of *Narcissus hispanicus* Gouan in the origin of *Narcissus bujei* and of cultivated trumpet daffodils (Amaryllidaceae). *Anales del Jardín Botánico de Madrid* 67(1): 29–39.