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GENOME SIZE, LEAF, FRUIT AND SEED TRAITS – TAXONOMIC TOOLS FOR SPECIES IDENTIFICATION IN THE GENUS *NASTURTIUM* R. BR.

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Flow cytometry estimation of 2C nuclear DNA content of the examined *Nasturtium* species resulted in taxonomic identification of *N.* × *sterile* in eight new localities, *N. microphyllum* in four new localities and *N. officinale* in one new locality in western Poland. Scanning electron microscopy proved a few of the micromorphological traits of seeds and fruits (size and shape of cells on the fruit septum surface, their anticlinal walls; secondary sculpture on the outer periclinal walls of cells on the siliqua valve internal surface) to be of taxonomic importance.

Key words: *Nasturtium microphyllum*, *N.* × *sterile*, *N. officinale*, flow cytometry, fruit, seed micromorphology.

INTRODUCTION

The genus *Nasturtium* R. Br. (Brassicaeae, tribe Cardamineae) with five species (Al-Shehbaz and Price, 1998; Al-Shehbaz et al., 2006) in Central Europe is represented by three taxa: the tetraploid *N. officinale* R. Br. (2n = 4x = 32), the octoploid *N. microphyllum* (Boenn. ex Rchb.) (2n = 8x = 64), and its hexaploid hybrid *N.* × *sterile* (Airy Shaw) Oefel. (2n = 6x = 48). *N. officinale* is native to southern Europe and most of Atlantic parts of Central Europe, while *N. microphyllum* is distributed in the northern parts of Central Europe. Both species are self- and cross-fertile. The formation of the hybrid *N.* × *sterile* has been favored by bringing *N. officinale* into the natural distribution area of *N. microphyllum* and by cultivation of the hybrid (Hurka et al., 2003). In Poland, *N. officinale* is a rare species and reaches the limit of the European range. However, most of the sites were reported before 1945 (Spałek, 2012). *N. microphyllum* is also rather uncommon, while *N.* × *sterile* is presently known from only one locality in the middle-west of Poland (Czarna and Morozowska, 2009; 2012; Czarna et al., 2013).

The taxonomic identification of *Nasturtium* species is currently based on seeds and fruits morphology; however, it is problematic since these plants do not bloom, so they do not set fruits or seeds every season. The hybrid species *N.* × *sterile*, which is widely distributed in Central Europe, has been notoriously overlooked or misidentified as *N. microphyllum* (Bleeker et al., 1997). These difficulties show the need for taxonomic verification or description of the existing and new localities of *Nasturtium* species with the use of genome size evaluation.

The aims of the presented study were as follows: (1) genome size estimation and taxonomic identification of *Nasturtium* species in the examined localities and (2) examination of leaves morphology as well as seeds and fruits micromorphology of the

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TABLE 1. List and collection data of populations of *Nasturtium* species examined by FCM.

Species	Site localization/province	Specimen collection data	Fruiting phase
*Nasturtium officinale**	Dubie, Szklarka river/ Małopolska	50°09'09" N 19°41'34" E leg. Aneta Czarna, 2011	+
*Nasturtium ×sterile**	Biała, ditch, an affluent of the Noteć river /Wielkopolska	53°02'44" N 16°32'23" E leg. Aneta Czarna, 2008	+
*Nasturtium microphyllum**	Zieleniewo, Wielki Rów, an affluent of the Parsęta river/Zachodnio-Pomorskie	54°06'25" N 15°33'43" E leg. Aneta Czarna, 2011	+
Nasturtium microphyllum	Babimost, Leniwa Obra river/ Lubuskie	52°09'52" N 15°49'38" E leg. Aneta Czarna, 2011	–
Nasturtium officinale	Głogów, Czarna river/ Dolnośląskie	51°39'36" N 16°05'04" E leg. Dajdok 2006	–
Nasturtium officinale	Przedmoście, ditch, an affluent of the Czarna river/ Dolnośląskie	51°37'42" N 16°09'41" E leg. Aneta Czarna, 2011	–
Nasturtium microphyllum	Makrosice, drainage ditch/ Lubuskie	51°51'38" N 14°38'42" E leg. Aneta Czarna, 2008	+
Nasturtium microphyllum	Mielno, drainage ditch/ Lubuskie	51°48'03" N 14°39'28" E leg. Aneta Czarna, 2011	–
Nasturtium microphyllum	Gęzawa, drainage ditch/ Lubuskie	51°42'28" N 14°51'37" E leg. Aneta Czarna, 2011	–
Nasturtium microphyllum	Czerna, drainage ditch/ Lubuskie	51°31'54" N 15°14'11" E leg. Aneta Czarna, 2011	–
Nasturtium ×sterile	Kopanica, Północny canal of the Obra River/ Wielkopolska	52°05'41" N 15°55'09" E leg. Aneta Czarna, 2007	–
Nasturtium ×sterile	Jaromierz, Północny canal of the Obra river/Wielkopolska	52°05'57" N 15°57'04" E leg. Aneta Czarna, 2011	–
Nasturtium ×sterile	Nowa Wieś, Południowy canal of the Obra river/Wielkopolska	52°00'48" N 16°12'47" E leg. Aneta Czarna, 2011	–
Nasturtium ×sterile	Kościan, Kościański canal/ Wielkopolska	52°04'56" N 16°38'18" E leg. Aneta Czarna, 2011	–
Nasturtium ×sterile	Kębłowo, Północny canal of the Obra river/Wielkopolska	52°02'53" N 16°06'06" E leg. Aneta Czarna, 2011	–
Nasturtium ×sterile	Świętno, Południowy canal of the Obra river/ Wielkopolska	52°00'31" N 16°03'03" E leg. Aneta Czarna, 2011	–
Nasturtium ×sterile	Obra, Północny canal of the Obra river/ Wielkopolska	52°04'27" N 16°02'40" E leg. Aneta Czarna, 2011	–
Nasturtium ×sterile	Chwalim, Obrzyca river/ Lubuskie	52°03'59" N 15°49'17" E leg. Aneta Czarna, 2011	–

*plant material (leaves, fruits and seeds) from marked populations was examined also by biometrical measurements (see Table 2)

studied *Nasturtium* species in order to find some new taxonomic features.

MATERIALS AND METHODS

Plant material including either leaves and/or fruits and seeds was collected in the field or obtained from herbarium vouchers in 2007–2014 from 35 populations of the tested *Nasturtium* species localized in western and southern parts of Poland and in several European countries (Tab. 1 and Tab. 2).

FLOW CYTOMETRY

Flow cytometry (FCM) analysis was performed on leaves collected from 18 of the examined populations of *Nasturtium* species growing in Poland (Tab. 1). In all localities *N. officinale*, *N. microphyllum* and *N. × sterile* plants grew in optimal habitats and occurred exclusively in monospecious phytocoenoses forming bigger or smaller patches floating on the water surface near rushes.

Each population was represented by five specimens and the collected plant material included the

TABLE 2. List and collection data of *Nasturtium* species localizations examined by biometrical measurements.

Species	Specimen collection data	Plant material		
		Leaves	Fruits	Seeds
*Nasturtium ×sterile**	Poland; Wielkopolska province; leg. A. Czarna 2008	+	+	+
Nasturtium ×sterile	Poland; Wielkopolska, Pn. Kanał Obry; leg. A. Czarna 2007	–	+	+
Nasturtium ×sterile	Russia; Not. Syst. Herb. Inst. Bot. Acad. Sci. USSR, leg. H. Grosorobeknij 1927; rev. A. Czarna 2013	–	+	+
Nasturtium ×sterile	Poland; Zachodnio-Pomorskie province leg. K. Krause 1855; rev. A. Czarna 2013	–	+	+
*Nasturtium officinale**	Poland; Małopolska province; leg. A. Czarna 2011	+	+	+
Nasturtium officinale	Poland; Herb. des Bot. Gart. zu Breslau, leg. K. Aust 1877	–	+	+
Nasturtium officinale	Romania; Collect. Flora Romania Exciccata, leg. A. Coman 1939; rev. A. Czarna 2013	–	+	+
Nasturtium officinale	Poland; Pieniny Mountains; leg. A. Czarna 2009	–	+	+
Nasturtium officinale	Croatia; Stobreč (Split district); leg. B.E.E. Duyfjer, R. Hengeveld & C.W. van der Voof 1963	–	+	+
Nasturtium officinale	Poland; Małopolska province; leg. A. Czarna 2011	–	+	+
Nasturtium officinale	Poland; Wielkopolska province; leg. K. Latowski 1969, rev. A. Czarna 2012	–	+	+
Nasturtium officinale	Italy; Rome's Botanical Garden; University of Sapienza; sample no 127001 005/09/03/10	–	+	+
Nasturtium officinale	Spain; Asturias leg. J.N. Compoaner & J.A. Molina Abril 1997; (Conservatoire et Jardin Botaniques, Ville de Genève, sheet no G00443491	–	+	+
*Nasturtium microphyllum**	Poland; Zachodnio-Pomorskie; leg. A. Czarna 2011	+	+	+
Nasturtium microphyllum	Poland; Lubuskie province; leg. A. Czarna 2011	–	+	+
Nasturtium microphyllum	Poland; Lubuskie province; leg. A. Czarna 2011	–	+	+
Nasturtium microphyllum	Poland; Pomorskie province; leg. no data (TRN)	–	+	+
Nasturtium microphyllum	Poland; Pomorskie province; leg. H. Ross 1881; rev. A. Czarna 2013 (POZG)	–	+	+
Nasturtium microphyllum	France; Anty, dèp. Des Ardennes; leg. J. Duvigneaud 1976 (TRN)	–	+	+
Nasturtium microphyllum	France; Anty, dèp. Des Ardennes; leg. J. Duvigneaud n° 8343 (Conservatoire et Jardin Botaniques, Ville de Genève, sheet no G00443471)	–	+	+

*plant material (fresh leaves) from marked populations was examined also by FCM (see Table 1)

upper fully grown leaves excised from plants growing at a distance of not less than five meters from each other. Leaves of *Petunia hybrida* P×Pc6 (2C=2.85 pg; Marie and Brown, 1993) were used as an internal standard. Nuclei suspensions were obtained after chopping off approximately 0.5 cm^2 of leaf tissue, according to Galbraith et al. (1983). Plant tissues from the targeted species and internal standard were prepared as described previously by Morozowska et al. (2010). Nuclear DNA content was measured in five replicates for each population of *Nasturtium* genus. Histograms were analyzed using FloMax software (Partec GmbH). The genome size was calculated as the target peak mean, divided by the internal standard peak mean, and multiplied by the amount of 2C DNA of P. hybrida standard. The coefficients of variation of the 2C DNA content were estimated for all the samples of the studied *Nasturtium* species. The 1C genome size was calculated after the conversion of values in picograms into base-pair numbers using the formula 1 pg = 978 Mbp (Doležel and Bartoš, 2005).

MICROMORPHOLOGY AND STATISTICAL ANALYSIS

Biometrical measurements were performed on fruits and seeds of plant material collected from 20 populations, and on leaves which originated from three populations among all the examined *Nasturtium* species localities (see Tab. 1 and Tab. 2). For each of the examined localities a sample of five mature fruits or 30 leaves was analyzed. In regard to seeds, for each population of *N. officinale* and *N. microphyllum* seed samples consisted of ten well-developed seeds, while for *N.* × *sterile*, due to a poor seed set of the hybrid species, seed samples consisted of 3–9 seeds, depending on their availability. The following fruits and seeds micromorphological characters were measured: length and width of seed surface cells, septum surface cells and siliqua valve internal surface cells, number of the transverse secondary striations present on periclinal walls of the cells on the siliqua valve internal surface. All measurements were performed with the use of scanning electron microscopy (SEM) for 10 cells per each fruit and seed. Seeds and fruits were dried up prior to observations using an acetone sequence at the following concentrations: 30, 50, 70, 90 and 100%, three times for six minutes each. Next they were covered with gold and examined with a Zeiss EVO 40 electron microscope at 8–15 kV, depending on the species. Barthlott's (1981) terminology was applied to describe fruit and seed micromorphology.

The following features of leaves were measured: leaf length, top leaflet length and width, distance from the top leaflet to the first pair of leaflets, number of leaflets, 1-st leaflet length and width, 2-nd leaflet length and width.

The one-way analysis of variance (ANOVA) was carried out to verify the hypothesis about lack of differences between *Nasturtium* species for all the examined morphological traits. The one-way analysis of variance, for *N. microphyllum*, *N. officinale*, and *N.* × *sterile* independently, was carried out to verify the hypothesis about lack of differences between seeds and fruits for the studied traits. The mean values and standard deviations (sd) were calculated. The least significant differences (LSDs) for each trait were estimated and homogeneous groups were determined. Euclidian similarity (estimated on the basis of all the examined morphological traits) was used as the similarity coefficients for cluster analysis with the unweighted pair group arithmetic means method (Skomra et al., 2013). Data analysis was performed using the statistical package GenStat release 10.1 (2007). The results of nuclear DNA content were analyzed using a Scheffe's test in STATISTICA 10.0 (StatSoft). The specimens and seed materials collected in the field are deposited in the herbarium of the Botany Department (POZNB), Poznan University of Life Sciences, Poland.

RESULTS

FLOW CYTOMETRY

On the basis of the flow cytometry estimation of 2C nuclear DNA content, eight new localities of *N.* × *sterile*, four new localities of *N. microphyllum* and one new locality of *N. officinale* in Poland were described. Additionally, the verification of the presence of *Nasturtium* spp. in few historical localities described in literature or in herbarium materials was done. In population from Dubie where, according to Tacik (1985), the presence *N.* × *sterile* was described, the obtained FCM results (0.770 pg 2C DNA content) proved the presence of *N. officinale* in that location. Similarly, in the population from Mielno (KRAM, Mielno, 19.06.1963, leg. M. Cieciura) *N. officinale* plants were described, while the estimated 2C DNA content (1.453 pg) proved the presence of *N. microphyllum* plants. The obtained results also confirmed some earlier taxonomic identifications concerning the presence of *N. officinale* plants in the population localized in Głogów (Dajdok and Nowak, 2006) as well as the presence of *N.* × *sterile* and *N. microphyllum* plants in the populations of Biała and Makrosice, respectively (Morozowska et al., 2010).

Most of the obtained histograms of 2C DNA content were good quality as the coefficient of variation was less than 5% (Galbraith et al., 2002). Some of them contained two peaks. The first distinct peak corresponded to nuclei arrested in the G_0/G_1 phase of the cell cycle, and a very small or even not detectable second peak corresponded to the G_2 phase (Fig. 4). The results of the nuclear DNA content of the studied species are shown in Table 3. No statistically significant variation in DNA measurements was observed among any population of the analyzed species. The 2C DNA content determined for plants of tetraploid species *N. officinale* ranged from 0.760 to 0.776 pg. The mean 2C DNA value for the octoploid genotype *N. microphyllum* was 1.460 pg while the genome size estimated for the hexaploid hybrid *N.* × *sterile* was more-or-less midway between that of the tetraploid and octoploid species, and it ranged from 1.121 to 1.140 pg/2C (Table 3).

MORPHOLOGICAL CHARACTERS

The results of one-way analysis of variance (ANOVA) indicated that the main effects of *Nasturtium* species were significant for all the examined morphological traits ($P<0.05$). The biometric measurements of the leaves revealed that the feature which differed significantly among all the examined species was the top leaflet width. Its average value was the lowest in *N. microphyllum*, the highest in *N. officinale* and intermediate in *N.* × *sterile* leaves. The

TABLE 3. Nuclear DNA content of 18 populations of *Nasturtium* species. The values are given as mean and standard deviation of the mean (SD) of the nuclear DNA content (pg/2C) and as the mean of the 1C genome size in Mbp.

Taxon	Population	2C DNA content (pg, mean ± SD)	Mean of 2C DNA for the taxon	1C genome size (Mbp) for the taxon
N. officinale	Głogów	0.760 ± 0.002^{ns}	0.771 ± 0.010c	377
	Dubie	0.770 ± 0.004		
	Przedmoście	0.776 ± 0.014		
N.×sterile	Jaromierz	1.121 ± 0.010^{ns}	1.127 ± 0.006b	551
	Kopanica	1.122 ± 0.012		
	Nowa Wieś	1.123 ± 0.005		
	Biała	1.125 ± 0.004		
	Obra	1.127 ± 0.009		
	Kębłowo	1.128 ± 0.013		
	Chwalim	1.129 ± 0.007		
	Świętno	1.130 ± 0.009		
	Kościan	1.140 ± 0.019		
N. microphyllum	Babimost	1.452 ± 0.017^{ns}	1.460 ± 0.008a	714
	Mielno	1.453 ± 0.016		
	Czerna	1.459 ± 0.018		
	Makrosice	1.468 ± 0.013		
	Gęzawa	1.468 ± 0.013		
	Zieleniewo	1.468 ± 0.017		

2C DNA values for the species followed by different letters are significantly different at $P<0.05$; ns: no significant difference between populations.

average values of the leaf length and the top leaflet length were significantly higher in *N. officinale* leaves in comparison to the two other species, while *N. microphyllum* and *N. × sterile* did not differ from each other with regard to these traits. Two features which significantly differentiated leaves of *N. microphyllum* from leaves of *N. officinale* and *N. × sterile* were the distance from the top leaflet to the first pair of leaflets (the lowest value) and the number of leaflets (the highest value). For *N. officinale* and *N. × sterile* leaves these two traits did not differ from each other. The average values of the length and width of the 1-st and the 2-nd pair of leaflets were significantly higher for *N. offcinale* comparing to the two other examined species, with an exception of the 2-nd pair of leaflets length, which was similar in *N. offcinale* and *N. microphyllum*. Almost no differences were found between the size of the 1-st and the 2-nd pair of leaflets in *N. × sterile* and *N. microphyllum* leaves (Tab. 4).

With respect to all examined micromorphological features concerning seeds and fruits, the differences in mean values of particular traits were found to be significant among the three examined *Nasturtium* species (Tab. 4). Seed coat sculpturing of the examined *Nasturtium* species was of reticulate type. Seed surface cells were tetra- and polygonal in shape with raised, straight to sometimes slightly undulate, rod-like anticlinal walls. Undulation of anticlinal walls was the strongest in cells on the surface of *N. × sterile* seeds. Periclinal walls were flat and the presence of the delicate reticulate-ocellate secondary sculpture pattern was detected. It was the most distinct on the surface of seeds of the hybrid species. On the anticlinal walls of testa cells the delicate transverse and longitudinal cuticle striations were visible and they were the most numerous and sharply outlined on *N. × sterile* seeds. Such striations were also present on the periclinal walls of seed coat cells of the hybrid species (Fig. 1). The average values of testa cells width were the lowest for *N. × sterile*, while the average values of testa cells length for the hybrid species were intermediate between the corresponding values of both parental species (Tab. 4).

Fruit septum surface sculpturing was reticulate in all the examined species. Anticlinal cell walls were straight to sinuate for *N. microphyllum*, straight for *N. officinale* and straight or slightly sinuate for *N. × sterile* fruits. In fruits of *N. microphyllum* and *N. officinale* the septum surface cells were strongly elongated and the average values of

TABLE 4. Mean values and standard deviations (s.d.) for observed traits of examined *Nasturtium* species.

Trait		*Nasturtium microphyllum*	*Nasturtium officinale*	*Nasturtium* × *sterile*	$LSD_{0.05}$	*F* statistic
Leaf length	Mean	11.840 b	13.631 a	11.634 b	1.281	5.85**
	s.d.	2.747	2.029	2.651		
Top leaflet length	Mean	3.363 b	5.443 a	3.753 b	0.4148	56.13***
	s.d.	0.554	0.999	0.809		
Top leaflet width	Mean	2.273 c	3.717 a	2.913 b	0.3123	42.37***
	s.d.	0.581	0.675	0.564		
Distance from the top leaflet to the first pair of leaflets	Mean	0.917 b	1.167 a	1.220 a	0.143	10.13***
	s.d.	0.273	0.291	0.272		
Number of leaflets	Mean	6.733 a	5.833 b	6.033 b	0.635	4.38*
	s.d.	1.337	1.234	1.129		
1-st leaflet length	Mean	2.830 b	3.393 a	2.637 b	0.2924	14.28***
	s.d.	0.623	0.452	0.618		
1-st leaflet width	Mean	1.593 b	1.913 a	1.573 b	0.1806	8.82***
	s.d.	0.406	0.322	0.321		
2-nd leaflet length	Mean	2.453 a	2.487 a	2.070 b	0.32	4.14*
	s.d.	0.617	0.585	0.666		
2-nd leaflet width	Mean	1.433 b	1.747 a	1.427 b	0.1836	7.83***
	s.d.	0.349	0.343	0.381		
Seed surface cell width	Mean	66.89 c	133.43 a	70.97 b	2.966	1578.77***
	s.d.	14.64	31.24	20.31		
Seed surface cell length	Mean	109.10 b	205.60 a	97.90 c	4.617	1582.61***
	s.d.	22.22	49.76	25.96		
Septum cell length	Mean	173.73 a	166.41 b	75.01 c	7.25	398.09***
	s.d.	53.84	36.90	25.31		
Septum cell width	Mean	35.180 b	45.180 a	21.820 c	1.698	366.70***
	s.d.	9.869	10.851	7.260		
Siliqua valve cell length	Mean	93.05 a	69.67 b	53.35 c	3.015	334.11***
	s.d.	21.13	12.44	18.34		
Siliqua valve cell width	Mean	20.433 a	18.731 b	13.020 c	0.932	120.80***
	s.d.	6.172	5.209	4.153		
Number of striations	Mean	5.983 b	4.166 c	11.940 a	0.3751	848.68***
	s.d.	1.509	0.791	3.905		

*P<0.05; **P<0.01; ***P<0.001. In rows, means followed by the same letters are not significantly different

their length were the highest in fruits of *N. microphyllum*. In *N.* × *sterile* fruits these cells were significantly shorter and narrower, and often rhombic in shape. The average values of width of the septum surface cells were the highest in *N. officinale* fruits (Table 4; Fig. 2).

The micro-ornamentation pattern on the siliqua valve internal surface of the examined *Nasturtium* species was reticulate. The outer cell boundaries were well defined and raised anticlinal walls were straight in *N. microphyllum* and *N. officinale*. In *N. microphyllum* fruits the cells on the siliqua valve internal surface were the broadest and longest with acute or rounded endings, while in *N. officinale* fruits these cells were narrower and shorter with straight, slightly acute or rounded endings. Outer periclinal walls were in both species a little concave with distinct transverse secondary striations (app. 4–6), and delicate cuticle wrinkles present on their surface. The internal surface of siliqua valve in *N.* × *sterile* fruits was characterized by the shortest and narrowest cells with the highest number of sec-

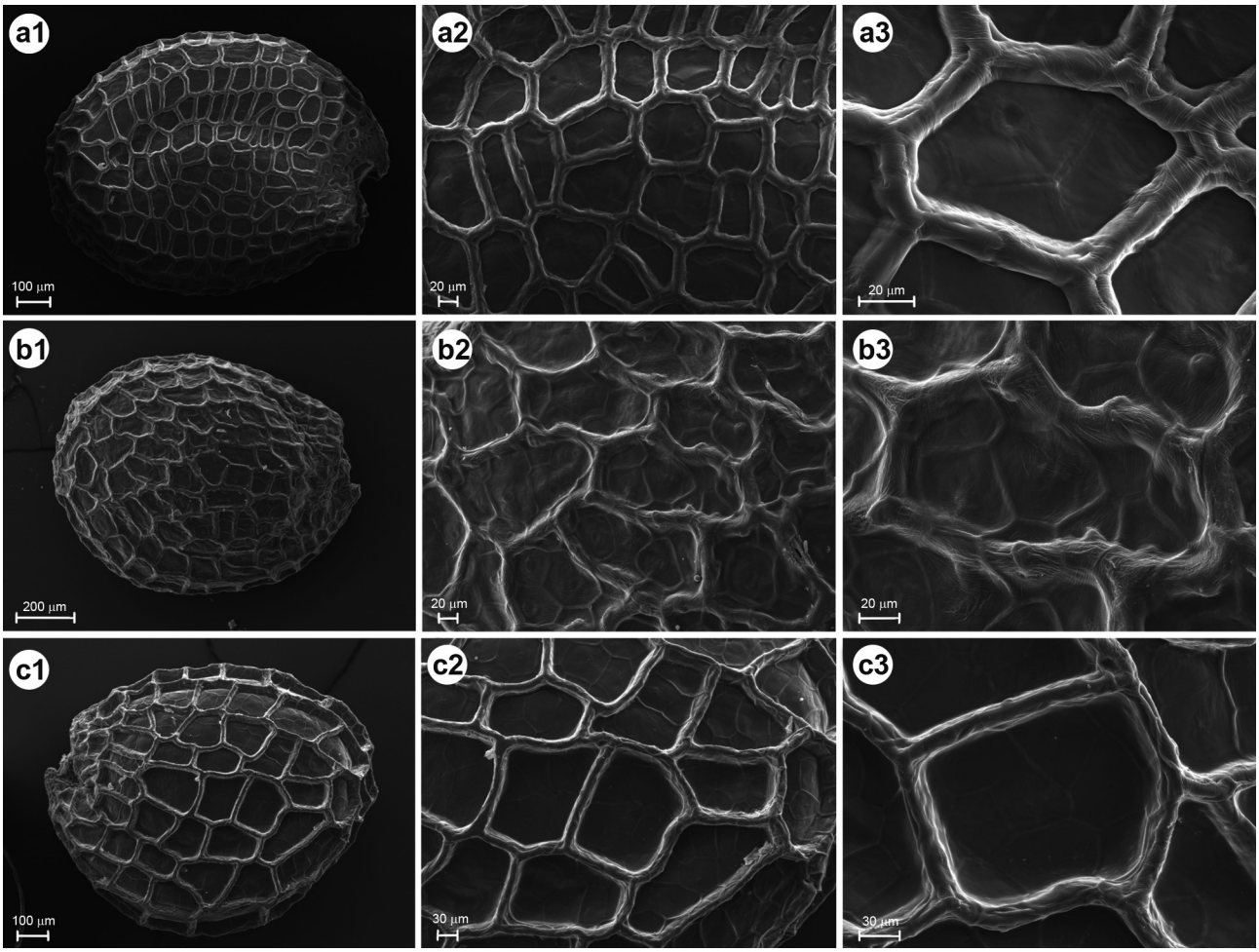

Fig. 1. Seed morphology of (**a1–a3**) *Nasturtium microphyllum*, (**b1–b3**) *N.* × *sterile*, and (**c1–c3**) *N. officinale*. (**a1**) Seed outline, (**a2**) Micro-reticulate primary and secondary sculpturing with straight cell walls visible, (**a3**) Channeled cell walls of secondary reticulate-ocellate pattern and slight cuticle striations visible on anticlinal walls, (**b1**) Seed outline, (**b2**) Micro-reticulate primary and secondary sculpturing with slightly sinuate cell walls visible, (**b3**) Raised cell walls of secondary reticulate-ocellate pattern and numerous cuticle striations visible on anticlinal and periclinal walls, (**c1**) Seed outline, (**c2**) Micro-reticulate primary and secondary sculpturing with mainly straight cell walls visible, (**c3**) Slightly raised cell walls of secondary reticulate-ocellate pattern and weak cuticle striations visible on anticlinal walls.

ondary transverse striations (>11). Due to the presence of the numerous secondary striations in *N.* × *sterile* fruits, the outer-cell boundaries on the siliqua valve internal surface were not very well-defined (Tab. 4; Fig. 2). The relationship between the examined species based on cluster analysis proved closer similarity of the hybrid to *N. officinale* than to *N. microphyllum* (Fig. 3). Euclidian distance between *N. microphyllum* and *N. officinale* was equal to 0.416, between *N. microphyllum* and *N.* × *sterile* it was equal to 0.582, and between *N. officinale* and *N.* × *sterile* it equaled 0.278.

The results of the one-way analysis of variance, for the species independently, indicated significant differences among all the measured seed and fruit parameters (Tab. 5).

DISCUSSION

Flow cytometry is a reliable method of species identification in the Nasturium genus when the fruits and seeds are not available (Morozowska et al., 2010; Czarna et al., 2013). The performed determination of 2C nuclear DNA content for genotypes of *Nasturtium* plants existing in vegetative stage cleared their taxonomic status, while for the genotypes of fructifying plants the estimated genome size was congruent with their species affinity estimated on the basis of seeds and fruits morphology. The evaluated 1C genome size for *N. officinale*, *N.* × *sterile* and *N. microphyllum* reached 377, 551, and 713 Mbp, respectively. Our results of nuclear DNA content of the studied species were

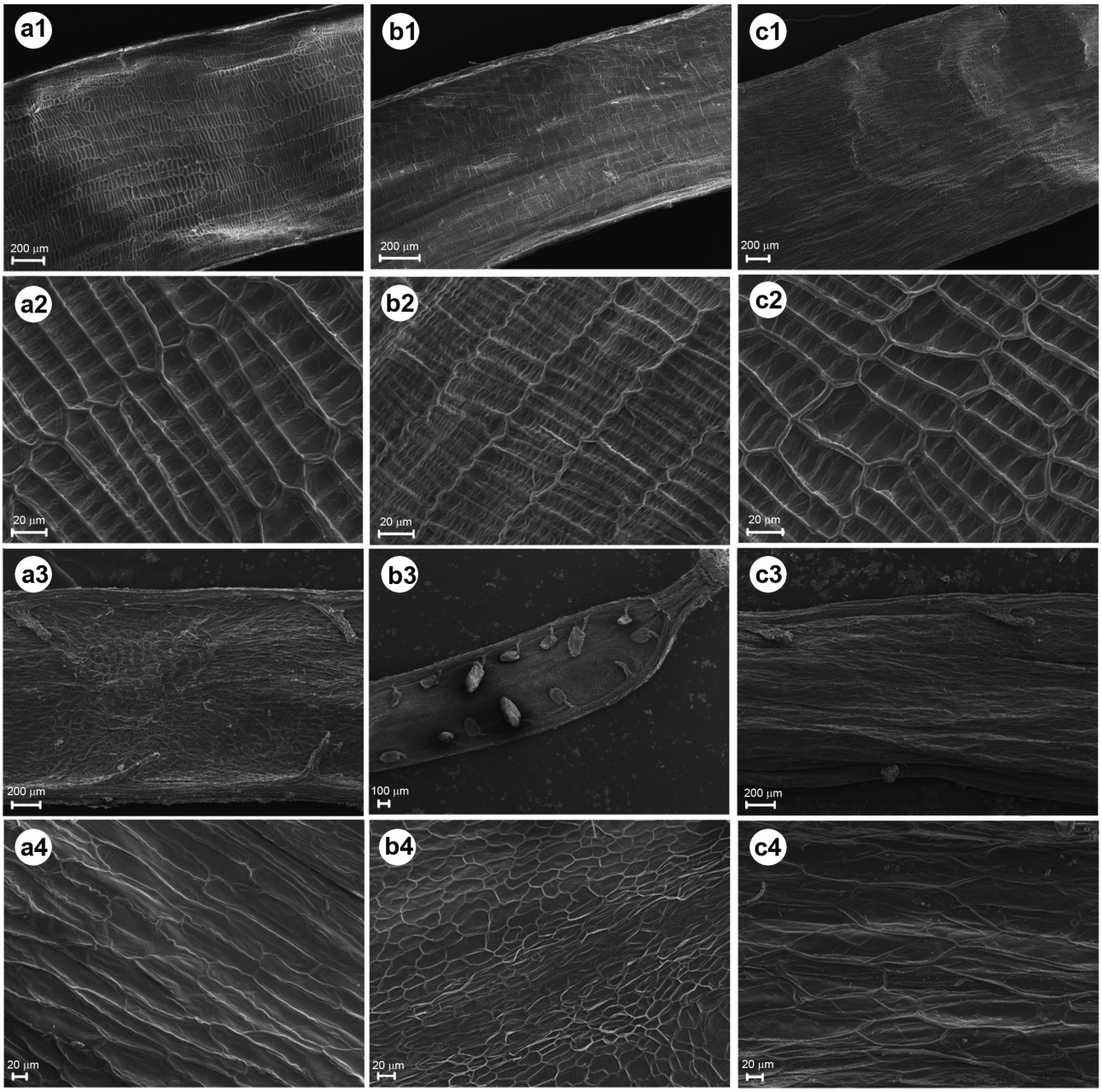

Fig. 2. Ultrastructure of siliqua valve and siliqua septum of (**a1–a4**) *Nasturtium microphyllum*, (**b1–b4**) *N.* × *sterile*, and (**c1–c4**) *N. officinale*. (**a1**) Siliqua valve, (**a2**) Micro-reticulate ornamentation of internal surface of valve with app. 6 secondary transverse striations and cuticle wrinkles present on periclinal walls, (**b1**) Siliqua valve, (**b2**) Micro-reticulate ornamentation of internal surface of valve with numerous secondary transverse striations and cuticle wrinkles present on periclinal walls, (**c1**) Siliqua valve, (**c2**) Micro-reticulate ornamentation of internal surface of valve with app. 4 secondary transverse striations and cuticle wrinkles present on periclinal walls, (**a3**) Siliqua septum, (**a4**) Micro-reticulate ornamentation of septum with sinuate anticlinal walls in elongated cells visible, (**b3**) Siliqua septum, (**b4**) Micro-reticulate ornamentation of septum with irregular cells shape, (**c3**) Siliqua septum, (**c4**) Micro-reticulate ornamentation of septum with straight anticlinal walls in very elongated cells visible.

similar to those reported previously by Morozowska et al. (2010).

SEM studies of the details of the fruit and seed surface structure within Brassicaceae proved in many cases that the type of sculpturing is of taxonomic significance (El-Naggar, 1996; Fayed and El-Naggar, 1996, 1988; Koul et al., 2000; El-Naggar, 2005; Maciejewska-Rutkowska et al., 2007; Kasem

TABLE 5. Mean squares from analysis of variance (ANOVA) for observed traits for species independently.

Species	*Nasturtium microphyllum*		*Nasturtium officinale*		*Nasturtium* × *sterile*	
Source of variation	Seeds	Residual	Seeds	Residual	Seeds	Residual
Degrees of freedom	59	540	89	810	39	220
Seed surface cell length	1115.2***	425.7	5098***	2188	1776.9***	905.4
Seed surface cell width	572.6***	175.3	2382.0***	821.3	831.8***	386.8
Source of variation	Fruits	Residual	Fruits	Residual	Fruits	Residual
Degrees of freedom	29	270	34	315	19	180
Septum cell length	14419***	1662	5391.6***	926.5	3527.3***	335.9
Septum cell width	462.09***	58.24	487.54***	77.3	247.68***	32.12
Siliqua valve cell length	1914.9***	288.7	807.16***	84.28	2598.61***	97.56
Siliqua valve cell width	113.36***	30.01	126.15***	16.45	95.336***	9.002
Number of striations	9.868***	1.461	1.7114***	0.5086	41.64***	12.47

***P<0.001

et al., 2011; Kaya et al., 2011). Seeds of *Nasturtium* spp. are characterized by the reticulate sculpturing with a different number of cells on the seed surface varying from >130 to about 60 on one side of the seed, depending on the species (Bleeker et al., 1997; 1999; Naqinezhad, 2006; Haeupleur and Muer, 2007). More detailed studies of *N. officinale* seed sculpturing resulted in classifying it as microreticulate-type, subtype ocellate, with raised anticlinal and flat periclinal walls (El-Naggar, 2005). The same author also described the presence of some concentric secondary striations on periclinal walls of the seed surface cells. The results obtained in the presented study concerning *N. officinale* confirmed the type and the sub-type of testa sculpturing; however, the type of the secondary sculpture pattern described by El-Naggar (2005) was not observed. For *N. microphyllum* and *N.* × *sterile* seeds, testa micro-ornamentation pattern was described in this work for the first time. A few features of *N.* × *sterile* seed primary sculpture (undulation of anticlinal walls) and secondary sculpture (presence of the reticulate-ocellate pattern and transverse or longitudinal cuticle striations on periclinal walls) were intermediate in their character when compared to the seeds of the hybrid parental species. The size of cells on the seed coat surface of the three examined species was found to be significantly different. *N. officinale* seeds were characterized by the biggest cells present on the testa surface, in comparison with *N. microphyllum* and *N.* × *sterile* seeds. This is in agreement with the lowest (25–60) number of cells present on one side of the seed (Bleeker et al., 1997; 1999; Naqinezhad, 2006; Haeupleur and Muer, 2007). Micromorphological details of the siliqua valve internal surface and septum sculpturing of *Nasturtium* spp. were described in this work for the first time. The obtained results showed that the fruits of *N.* × *sterile* were characterized by the smallest cells on the surface of the fruit septum and on the siliqua valve internal surface. The examined *Nasturtium* species differed distinctly in the size and shape of cells on the septum surface and according to their anticlinal walls. Another trait that clearly distinguished the studied species was the number of secondary striations present on the outer periclinal walls of cells on the siliqua valve internal surface. These four characters were recognized as taxonomically important.

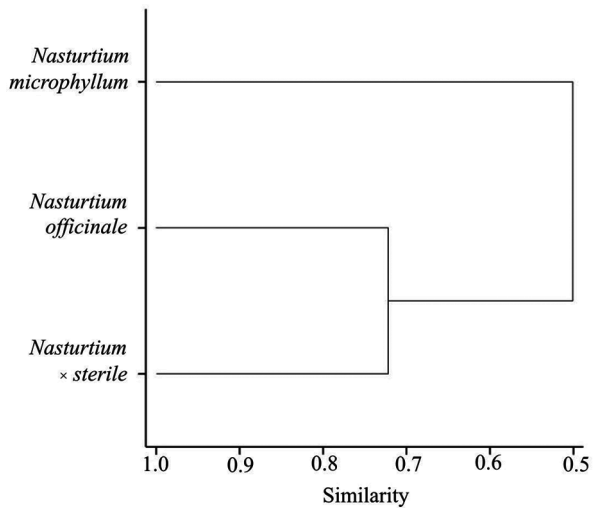

Fig. 3. Dendrogram hierarchical clustering of similarity of three *Nasturtium* species on the basis of the means of sixteen examined morphological characters (UPGMA).

Leaves of *Nasturtium* species are pinnate with (1) 3–9 (13) leaflets, which are different in size and shape and of great morphological variation. The results of the biometric measurements of the leaves showed some close similarities in leaf size among the examined *Nasturtium* species. Only one of all the

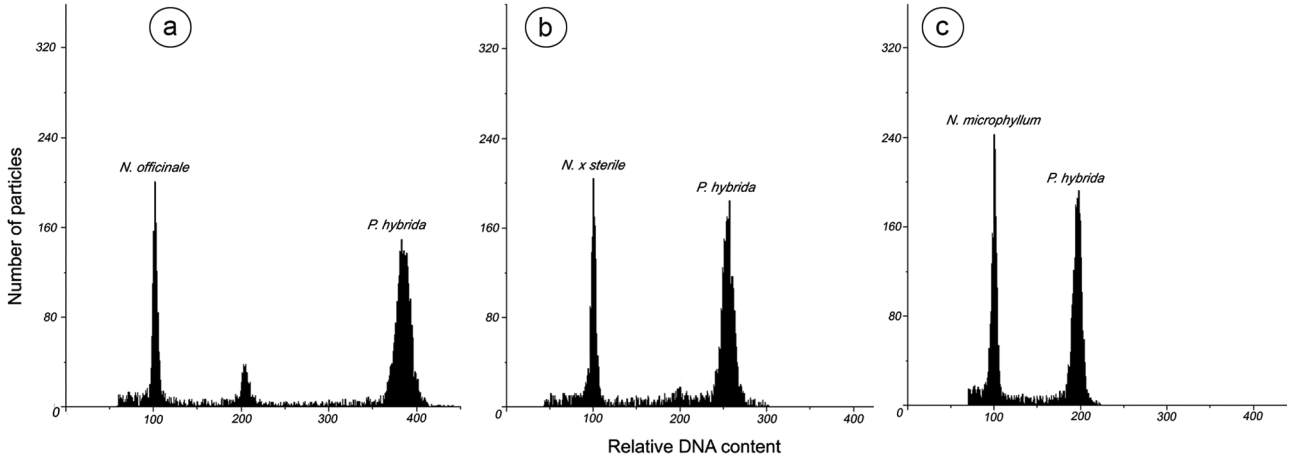

Fig. 4. Histograms of nuclear DNA content obtained after flow cytometry measurement of the PI-stained nuclei isolated from young leaves of *Petunia hybrida* (internal standard) and (**a**) *Nasturtium* officinale, (**b**) *Nasturtium* × sterile and (**c**) *Nasturtium microphyllum*.

analyzed characters, namely the top leaflet width, was found to significantly differentiate leaves of the studied genotypes. The results of the mutual comparisons of the remaining examined traits revealed that the average values of seven out of nine tested features were bigger for *N. officinale* leaves in comparison with *N. microphyllum* and *N.* × *sterile* leaves. Leaves of *N. microphyllum* and *N.* × *sterile* differed from each other only in four of all the investigated traits.

The dichotomous key for identifying *Nasturtium microphyllum*, *N. officinale*, and *N.* × *sterile* based on seed and fruit micromorphology is proposed.

1. Seed surface epidermal cells with distinct secondary reticulate-ocellate micro-ornamentation pattern on periclinal walls, cuticle striations present on anticlinal and periclinal walls; number of secondary transverse cuticle striations on periclinal walls of epidermal cells on siliqua valve internal surface ≥ 8; siliqua septum epidermal cells ≤ 100 μm long.. .... *Nasturtium* × *sterile*

1.* Seed surface epidermal cells with weakly visible secondary reticulate-ocellate micro-ornamentation pattern on periclinal walls, cuticle striations present only on anticlinal walls; number of secondary transverse cuticle striations on periclinal walls of epidermal cells on siliqua valve internal surface < 8; siliqua septum epidermal cells > 100 μm long 2

2. Number of secondary transverse cuticle striations on periclinal walls of epidermal cells on siliqua valve internal surface > 4; anticlinal walls of septum epidermal cells often undulate ..... *N. microphyllum*

2.* Number of secondary transverse cuticle striations on periclinal walls of epidermal cells on siliqua valve internal surface ≥ 4; anticlinal walls of septum epidermal cells straight .... *N. officinale*

CONCLUSION

The evaluation of DNA content was successfully used for identifying *Nasturtium* spp. plants, especially when they do not bloom and produce fruits. SEM examination of micromorphological traits of seeds and fruits allowed to find a few new taxonomically important characters such as the size and shape of cells on the septum surface, their anticlinal walls and the number of secondary striations present on the periclinal walls of cells on the siliqua valve internal surface. Flow cytometry combined with morphological assessment may be successfully used in plants taxonomic identification.

AUTHORS' CONTRIBUTIONS

We acknowledge that each of the authors has substantial contribution to our manuscript with respect to conception and design of the work, acquisition of data and analysis as well as interpretation of the obtained results. All of the authors approve the final version of the work to be published and there are no conflicts of interest.

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