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Wojciech Antkowiak, Irmina Maciejewska-Rutkowska, Iwona Jędrzejczyk, Andrzej Wojciechowski

Morphological, anatomical and cytological characteristics of spontaneous hybrid *Pyrus ×myloslavensis*

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Abstract: *Pyrus ×myloslavensis* is a hybrid with intermediate characteristics of leaves and fruits inherited from its parental species *P. salicifolia* and *P. communis*. The aim of this study was to determine whether these intermediate nature of this taxon would be visible in the pollen and seed anatomy and morphology. In addition, the authors evaluated self-compatibility and crossability based on the observation of pollen tube growth. The investigations were carried out using light, fluorescence and electron microscopies and flow cytometer. Based on pollen and seed morphology the current study confirmed a hybrid origin of the investigated taxon. Especially, pollen grains had most features similar to both parental species. In respect to epidermal micromorphology, the seeds of *P. ×myloslaviensis* were like *P. salicifolia*, but anatomical structure of the seed coat of the hybrid was more consistent with *P. communis*. The data obtained from the current experiment showed that *P. ×myloslavensis* was self-incompatible and cross-compatible only with one of four tested *P. communis* cultivars ('Conference') and with *P. salicifolia*. In turn, the average genome size of the hybrid was smaller than putative parents.

Keywords: pear, hybridization, seeds, pollen, genome

Addresses: W. Antkowiak, Department of Botany, Poznań University of Life Sciences, Wojska Polskiego 71 c, 60-625 Poznań, Poland
I. Maciejewska-Rutkowska, Department of Forest Botany, Poznań University of Life Sciences, Wojska Polskiego 71 d, 60-625 Poznań, Poland, e-mail: irminamr@up.poznan.pl
I. Jędrzejczyk, Department of Plant Genetics, Physiology and Biotechnology, UTP: University of Science and Technology, Kaliskiego 7 Ave., 85-796 Bydgoszcz, Poland
A. Wojciechowski, Department of Genetics and Plant Breeding, Poznań University of Life Sciences, Dojazd 11, 60-632 Poznań, Poland

Introduction

In 2008, within area of Wielkopolska province (western Poland), a new taxon of *Pyrus* (Rosaceae family), named *P. ×myloslavensis* Czarna & Antkowiak sp. hybr. nova was described (Antkowiak et al., 2008). Morphological analysis suggested a spontaneous hybrid occurred between *P. communis* L. and *P. salicifolia* Pall. The first putative parent is the most important commercial pear species cultivated in Europe

(including Poland). It is regarded to have hybrid origin, with *P. caucasica* Fed. and *P. pyraster* (L.) Burgsd. as its main wild ancestors. It evolved and was domesticated at two different centers, China and Asia Minor until the Middle East (Silva et al., 2014). Now more than a thousand cultivars of this species are known. At the same time *P. communis* is often naturalized and it is an important element of the rural landscape in Poland (Dolatowski et al., 2004). The natural range of *P. salicifolia* is limited to the Krym and European part of Turkey (Terpo & Amaral, 2010). In Poland it is an uncommon species planted as an ornamental tree, not observed to spread spontaneously. Different pear species, including *P. communis* and *P. salicifolia*, easily hybridize and more than 40 interspecific pear hybrids are known (e.g. Bell, 1986; Browicz, 1993). But according to the authors' knowledge, except *P. ×myloslavensis*, no other hybrid between those species has been reported in the literature.

Due to finding only a single individual of *P. ×myloslavensis* in the field, it had been propagated vegetatively. In 2009 the shoots of the hybrid were grafted onto *P. pyraster* rootstocks and 20 trees were planted in the experimental fields of the Department of Botany of Poznań University of Life Sciences (Poland). The first weak flowers were observed in 2013. although the next year flowering and fruiting was abundant. Having a sufficient number of flowers and seeds, it was decided to carry out comparative analyses of morphological and anatomical properties of pollen and seeds as well as genotype analyses of *P. ×myloslavensis* and its supposed parents (*P. salicifolia* and *P. communis* – selected cultivars). This study was initiated to identify intermediate features of the new hybrid species and to explore self-incompatibility and cross-compatibility based on the observation of pollen tubes. As it is known a consequence of cross compliance are likely new hybrids and increased gene pool.

Materials and Methods

Plant material

In the research plant material were *P. communis*, *P. salicifolia* and the hybrid *P. ×myloslavensis* F_1. Species *P. communis* was represented by four cultivars: 'Carola', 'Erica', 'Conference' and 'Concorde'. In palinological and carpological investigations the analyses were limited to only one cultivar 'Conference'. Leaves of *Petunia hybrida* (P×Pc6; 2.85 pg/2C, Marie & Brown, 1993) were used as an internal standard in flow cytometry analysis of genome size. Samples of seeds and pollen were gathered from the field: of *P. ×myloslavensis* from 20 trees cultivated in the collection of the Department of Botany of the Poznań University of Life Sciences, of *P. communis* from 10 trees for each cultivar cultivated in the collection of the Agriculture and Pomology Research Farm in Przybroda (Poznań University of Life Sciences) and of *P. salicifolia* from five trees growing in the Dendrological Garden of the Poznań University of Life Sciences, Botanical Garden of the Adam Mickiewicz University in Poznań and in the village of Nowa Wieś Podgórna near the road to Pyzdry (West Poland).

Palinological investigations

For light (LM) and scanning electron microscopy (SEM) samples of pollen grains were prepared according to the standard method described by Erdtman (1966). For LM the pollen grains were put into the drop of pure water and observations were made with a microscope Olympus BX 43. The measurements of 30 grains for each specimens were taken and the different traits were observed in polar and equatorial views (400× and 1000×). For SEM the samples of grains were mounted on aluminium stubs, coated with gold in a sputter coater, and examined with Leo scanning electron microscope, belonging to the Department of Biology Adam Mickiewicz University in Poznań (Poland). Pollen terminology presented by Punt et al. (2007) and Hesse et al. (2009) was used in this study.

Morphological and anatomical investigations of seeds

Before scanning electron microscopy (SEM) was done for morphological analysis of the seed coat, the seed samples were cleaned. Five seeds of each species were mounted on aluminium stubs, coated with gold in a sputter coater, and examined with Leo scanning electron microscope (Department of Biology, Adam Mickiewicz University in Poznań, Poland). Transverse sections in the middle of the seeds were made by microtome. The observations and measurements of seed coat anatomy of 15 seeds of each species were carried out with a microscope Olympus BX 43 (400× and 1000×). Terminology presented by Bojňanský and Fargašová (2007) and Beentje (2010) was used in this study.

Evaluation of self-incompatibility and cross-ability

Observations of pollen grain germination and pollen tube growth were made after self-pollination of *P. ×myloslavensis* and cross pollination of four cultivars of *P. communis* with two other investigated species. The pollination was carried out on cut branches

in glasshouse conditions. The pollen of particular pollinators was placed on the stigma of flowers which were emasculated at the bud stage. Pollinated pistils were collected 48 hours after pollination and fixed and stained with aniline blue according to Martin (1959) with modification of Wojciechowski (1985). Pollen grain germination and pollen tube growth were observed using UV-light fluorescence microscope.

Self-incompatibility (SI) or crossability (CC – cross compatibility, CI – cross incompatibility) were evaluated on the basis of the pollen grain germination index (PGI) according to Matsuzawa (1983) and modified by Wojciechowski (compare to Matsuzawa, additionally the number of pistils with the pollen tubes entering into the ovules was counted): PGI = (b+2c+3d+4e+5f) / (a+b+c+d+e+f), where: a – number of pistils with pollen grains, b – number of pistils in which pollen grains do not germinate, c – number of pistils in which pollen grains germinate on the stigma, d – number of pistils in which pollen tubes enter the style tissue, e – number of pistils in which pollen tubes penetrate ovary, f – number of pistils in which pollen tubes enter into the ovules. In case of PGI = or higher than 2 it was concluded that there was compatibility.

Flow cytometry analysis of genome size

The test for the presence of PI staining inhibitors in plant tissues was performed according the protocol of Price et al. (2000). Nuclei suspensions for genome size estimation were obtained according to the protocol described by Jędrzejczyk and Śliwińska (2010). Young and fresh leaves of the target species and of the internal standard were chopped with sharp razor blade in a plastic Petri dish containing 1 ml of nuclei isolation buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM 3-[N-morpholino] propanesulphonic acid, 0.1% v/v Triton X-100, pH 7.0) with the addition of propidium idioide (PI; 50 μg/mL) and ribonuclease A (RNase A; 50 μg/mL). Additionally buffer was supplemented with (w/v) polyvinylpyrrolidone (PVP-10). The suspension was passed through a 50-μm mesh nylon filter. The measurements of fluorescence intensities were performed in at least 7000 nuclei using CyFlow SL Green (Partec GmbH, Münster, Germany) flow cytometer, equipped with a laser with green light emission at 532 nm. Histograms were analyzed using the FloMax (Partec GmbH, Münster, Germany) software. Analyses were performed on ten replicates of each genotypes of *Pyrus*. Nuclear DNA content (in picograms, pg) was estimated using the linear relationship between the ratio of the 2C peak positions of studied species to internal standard on the histogram of fluorescence intensities. DNA content in pg was converted to Mbp by using the formula 1pg=978 Mbp (Doležel et al., 2003). Data were analyzed statistically using an one-way ANOVA followed by the Tukey test (STATISTICA 10.0 software).

Results

The investigated species are characterized by 3-zonocolporate, isopolar, radiosymmetric, medium size pollen grains (Tab. 1, Fig. 1). Grains of *P. ×myloslaviensis* are usually slightly larger than in the other spe-

Table 1. Details of pollen features of three investigated species in current study

Feature \ Species		*P. salicifolia*	*P. ×myloslaviensis*	*P. domestica*
Figure illustrated		1 A–D	1 E–H	1 I–L
Length of P axis (μm)		(30.00–) 35.07 (–40.00)	(32.00–) 37.03 (–44.00)	(30.00–) 35.77 (–40.00)
Length of E axis (μm)		(26.00–) 29.07 (–30.00)	(26.00–) 29.80 (–36.00)	(26.00–) 28.43 (–32.00)
P/E		(1.00–) 1.21 (–1.43)	(1.00–) 1.25 (–1.47)	(0.94–) 1.26 (–1.43)
Participation in shape classes (%)	oblate-spheroidal	–	–	2
	spheroidal	2	3	–
	prolate-spheroidal	18	37	8
	subprolate	55	50	65
	prolate	25	10	25
Exine thickness (μm)		(1.58–) 2.09 (–2.32)	(0.94–) 1.52 (–2.17)	(1.23–) 1.75 (–2.04)
Ratio of sexine thickness to nexine thickness		(0.9–) 1.1 (–1.3)	(0.6–) 0.8 (–1.0)	(0.8–) 1.0 (–1.2)
Colpus length (μm)		(26.3–) 30.8 (–37.9)	(28.8–) 33.3 (–39.6)	(28.7–) 33.4 (–39.0)
Ratio of polar axis length to colpus length		(1.06–) 1.09 (–1.14)	(1.0–) 1.11 (–1.25)	(1.0–) 1.05 (–1.12)
Colpus bridge		Single, rarely double	Single	Single, rarely double
Pattern of sculpturing of pollen grains		Striate	Striate	Striate
Ridge arrangement pattern		Both intersecting and parallel	Both intersecting and parallel	Parallel
Perforation number in one square of micrometer		0–3	0–3	1–4
Width of ridge (μm)		0.5–0.8	0.4–0.5	0.3–0.4

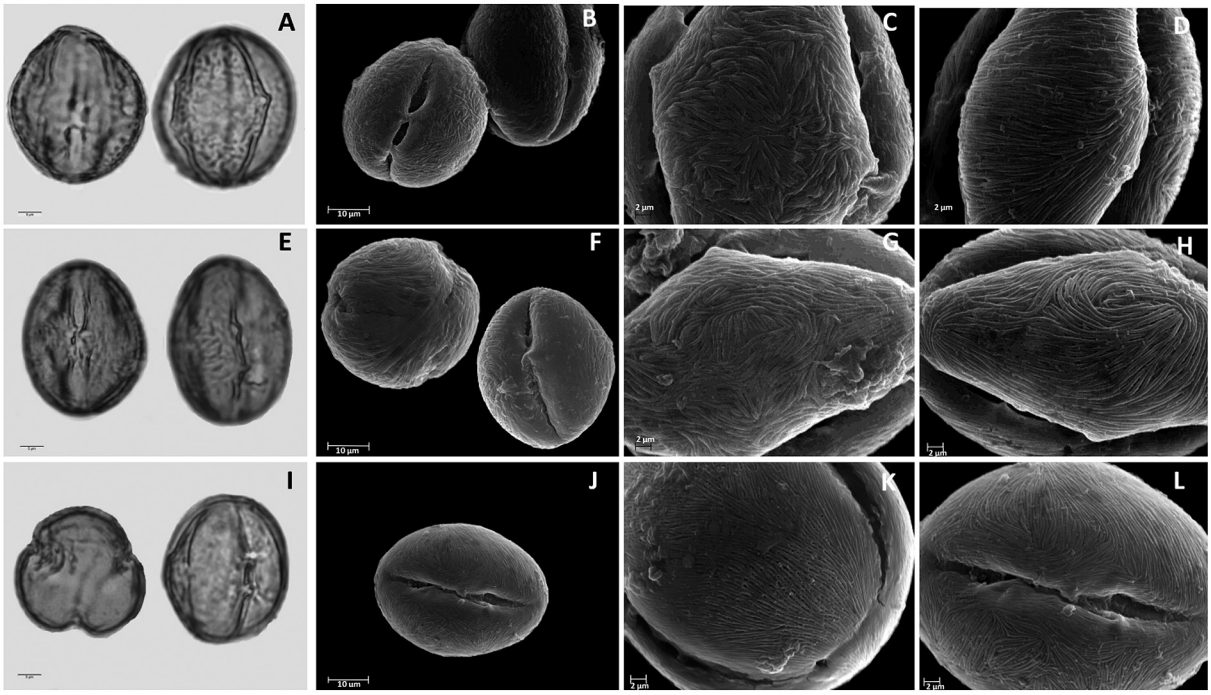

Fig. 1. Pollen morphology of studied *Pyrus* species: A-D *P. salicifolia*, E-H *P.* ×*myloslavensis*, I-L *P. communis* 'Conference': A) outline in equatorial view in LM, with the focus on equatorial bridge over colpus (left) and striate ornamentation of mesocolpium (right), B) outline in polar view in SEM, two equatorial bridges over one colpus visible, C) intersecting arrangement of striae in zone of mesocolpium (SEM), D) parallel arrangement of striae in zone of mesocolpium (SEM), E) outline in equatorial view in LM, with the focus on equatorial bridge over colpus (left) and striate ornamentation of mesocolpium (right), F) outline in polar and equatorial views in SEM, G) intersecting arrangement of striae in zone of mesocolpium (SEM), H) parallel arrangement of striae in zone of mesocolpium (SEM), I) outline in polar and equatorial views in LM, with the focus on two equatorial bridges over one colpus (right), J) outline in equatorial view in SEM, K) parallel arrangement of striae in zone of mesocolpium, with well marked perforations (SEM), L) parallel arrangement of striae in zone of apocolpium (SEM)

cies (- on average polar axis is ±2 μm longer). The outline of pollen grain in polar view is always semi-lobate, but pollen grains are variable regarding to their shape. In all species at least 50% of grains are subprolate, with the greatest participation of this shape class in *P. domestica*. Additionally, prolate-spheroidal type is very often observed in *P.* ×*myloslaviensis* and prolate type is relatively common in *P. salicifolia* and *P. communis* 'Conference'. Ectocolpus is rather long, on average a little shorter than the length of polar axis. In the middle of ectocolpus there is endoporus, covered by bridge. Sometimes two bridges over single ectoaperture are visible in *P. salicifolia* and *P. communis* 'Conference'. In general, pollen grains of *P.* ×*myloslaviensis* are similar to both putative parental species. However, they are usually slightly larger than other species (on average P axis is ± 2 μm longer). With respect of average value of P/E ratio, exine thickness, and width of stria ridge the pollen grains of *P.* ×*myloslaviensis* are more similar to *P. communis* 'Conference' than to *P. salicifolia*. While considering stria arrangement pattern and perforation density they are closer to *P. salicifolia*.

Exine is 0.9–2.3 μm thick, on average the thickest in *P. salicifolia* and the thinnest in *P. domestica*, but the differences among all species are small. Sexine and nexine are usually of similar thickness. All species are characterized by striate sculpture. Within area of mesocolpi the striae are elongate and parallel (-like finger-print arrangement) or shortened and intersecting in *P. salicifolia* and *P.* ×*myloslaviensis*. Except the direct zone of the ectocolpus only parallel striae are observed in *P. communis* 'Conference'. The widest striae are in *P. salicifolia* and the narrowest in *P. communis* 'Conference'. There are some perforations in the grooves, the most distinct in *P. communis* 'Conference'.

In all investigated species the cells of external epidermis of the seed are isodiametric, polygonal, most often pentagonal, less frequently hexagonal, with curved corners (Fig. 2 D, H, L). Anticlinal walls are arched or sometimes straight, rather thick, distinctly protrude, with the rounded edges. Periclinal walls are usually more or less concave, not often flat, completely convex; or flat with a convex central part. Epidermis is covered with a thin layer of cuticule, locally giving a striate ornamentation. Morphological differentiations of the outer epidermis among investigated species are not large and mainly reveal in a degree

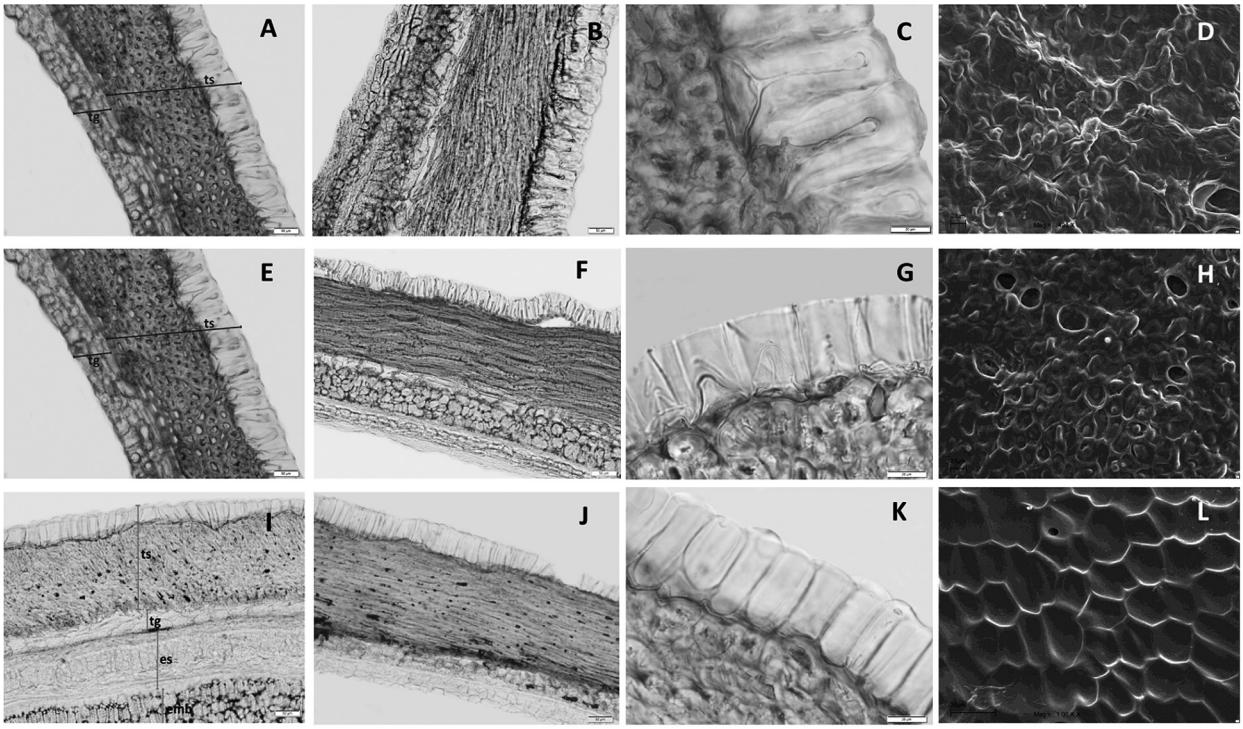

Fig. 2. Anatomical and morphological structure of seed coat of investigated *Pyrus* species: A–D *P. salicifolia*, E–H *P.* ×*myloslavensis*, I–L *P. domestica* 'Conference'. Column 1 – cross-sections of seed coat, 100× in LM; column 2 – longitudinal sections of seed coat, 100× in LM; column 3 – focus on epidermis in cross-section, 400× in LM; column 4 – surface of seed coat in SEM, 500× and 1000×. Abbreviations to column 1: ts – testa, tg – tegmen, es – endosperm, emb – embryo

of concavity of periclinal walls, irregularity of cell arrangement of testa surface and thickness of anticlinal walls. However, in respect to these epidermal features, the seeds of *P.* ×*myloslaviensis* are more similar to *P. salicifolia* than to *P. communis* 'Conference'. In seed micromorphology the first two species are characterized by highly diverse periclinal cells of external testa (from significantly concave to more or less convex) and by irregular ornamentation of the testa surface. On the other hand, the periclinal walls of *P. communis* 'Conference' are only shallowly concave and the testa cells form a regular arrangement on seed surface. In all investigated species the seed coat is more than 220 μm, however at most 390 μm thick (Tab. 2). On the average it is the thinnest in *P. communis* 'Conference' and the thickest in *P. salicifolia*. In cross section the layered structure of the seed coat is well marked, with distinct divisions into testa and tegmen (Fig. 2 A–C, E–G, I–K). Testa consists of outer epidermis and several layered mesophyll. The epidermis is composed by a layer of thick-walled, but not lignified, slightly elongated palisade cells, with the longest axis orientated perpendicularly to seed surface. Based on the anatomical structure of the seed coat, this layer is the most developed in *P. salicifolia*, with the thickest cell walls and at the same time the smallest cell lumina, as compared to other investigated species. Mesophyll consists of 9–14 layers of sclerotic cells longitudinally elongated. The innermost layer of it is strongly compressed. In comparison with the other two species average number of mesophyll layers of the testa is reduced by two or three in *P. salicifolia*. Mesophyll of tegmen consists of 2–3 layers of thin-walled cells, shortly, tangentially elongated. They are usually larger than adjacent cells of testa, but often crushed or partially so. The innermost layer of tegmen, separat-

Table 2. Details of anatomical features of seeds of three investigated species in current study

Feature \ Species	*P. salicifolia*	*P.* ×*myloslaviensis*	*P. domestica* 'Conference'
Thickness of the entire seed coat (μm)	(300–) 332 (–383)	(290–) 296 (–313)	(223–) 271 (–313)
Outer epidermis thickness (μm)	(66–) 81 (–93)	(40–) 43 (–50)	(33–) 48 (–58)
Thickness of mesophyll of testa (μm)	(133–) 159 (–190)	(183–) 202 (–213)	(167–) 187 (–210)
Entire thickness of testa (μm)	(207–) 215 (–280)	(217–) 238 (–280)	(200–) 234 (–267)
Number of mesophyll layers of testa	(9–) 10 (–12)	(10–) 12 (–14)	(10–) 13 (–14)
Thickness of tegmen (μm)	(37–) 65 (–100)	(40–) 53 (–67)	(27–) 37 (–50)
Number of mesophyll layers of tegmen	3	(2–) 3	2–3

Table 3. Self-incompatibility and cross-ability between three *Pyrus* species expressed by Pollen Germination Index (PGI)

Cross combination			
Maternal form	Paternal form		PGI
Pyrus ×*myloslavensis*	*Pyrus* ×*myloslavensis*		1.6*
	Pyrus communis:	'Carola'	1.0
		'Erica'	0.7
		'Conference'	2.1
		'Concorde'	0.7
	Pyrus salicifolia		2.1
Pyrus communis 'Conference'	*Pyrus salicifolia*		1.6
Pyrus salicifolia	*Pyrus communis* 'Conference'		2.3

*/ PGI < 2 means incompatibility (self or cross)

ing the seed coat from endosperm consists of small, flattened cells. On average the thickest tegmen is observed in *P. salicifolia* and the thinnest in *P. communis* 'Conference'. Based on the anatomical features of the seed coat, it seems that *P.* ×*myloslaviensis* is more related to *P. communis* 'Conference' than to *P. salicifolia*. In particular, the later species is distinguished by a more developed external epidermis of testa as well as a thicker seed coat.

The observation of pollen germination on the stigma and penetration rate of pollen tubes into the style and to the ovary showed that in the most pollination combinations Polen Germination Index (PGI) was lower than 2 (Tab. 3, Fig. 3). The new species *P.* ×*myloslavensis* proved to be self-incompatible (PGI = 1.6). In the crosses of that species with *P. communis* cultivars there were differences concerning PGI and only in the cross *P.* ×*myloslavensis* × *P. communis* 'Conference' the PGI was higher than 2 showing cross-compatibility (CC) between these two forms. Also in the crossing *P.* ×*myloslavensis* × *P. salicifolia* the CC was observed (PGI = 2.1). In the reciprocal crossing of *P. communis* and *P. salicifolia* there was unilateral CC observed. Only in this cross combination where *P. salicifolia* was used as maternal parent the cross-compatibility was observed (PGI = 2.3), while in crosses where *P. communis* 'Conference' was used as maternal form, cross-icompatibility was observed (PGI = 1.6).

The analysis of the nuclear DNA content of selected *Pyrus* genotypes yielded high resolution DNA his-

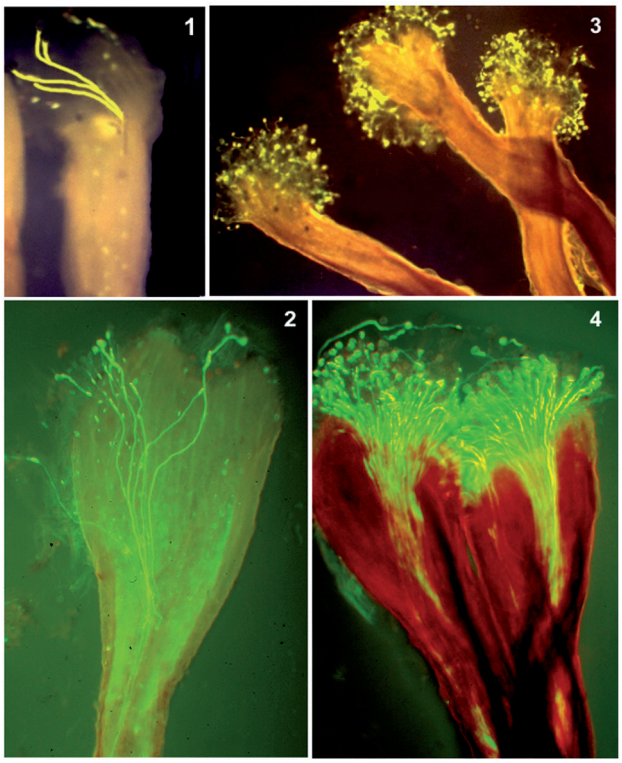

Fig. 3. Photos 1–2 – germinating pollen grains of *P.* ×*myloslavensis*, and pollen tubes blocked in the begining and in half of the style, 48h after self-pollination, photos 3–4 – pollen grains of *P. comunmunis* 'Conference' germinating on the stigma and penetrating into style and to the ovary of *P. salicifolia*, visible plenty of pollen grains forming pollen tubes on the three stigmas and pollen tubes entering into three styles of one pistil, 48h after cross-pollination

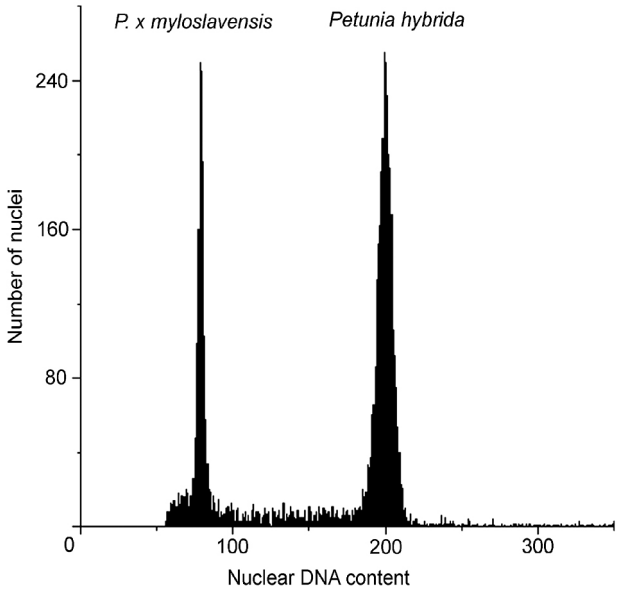

Fig. 4. Histogram of nuclear DNA content obtained after flow cytometric analysis of PI-stained nuclei isolated from the leaves of *P.* ×*myloslavensis* and internal standard, *Petunia hybrida*

Table 4. Genome size estimation of *Pyrus* genus

Species	Cultivars	Nuclear DNA content (pg/2C ± SD)	2C (Mbp)	CV of target species (%, mean)
Pyrus communis	'Erica'	1.143 ± 0.009 [a]	1118	4.82
	'Concord'	1.142 ± 0.006 [a]	1117	4.40
	'Carola'	1.139 ± 0.004 [a]	1114	4.32
	'Conference'	1.133 ± 0.014[ab]	1108	4.13
Pyrus salicifolia		1.126 ± 0.006[bc]	1101	4.36
Pyrus ×*myloslavensis* F_1		1.114 ± 0.013 [c]	1086	4.28

tograms (Fig. 4) with coefficients of variation ranging from 4.13 to 4.82%. Genome size of the investigated genotypes ranged from 1.114 to 1.143 pg/2C. The highest genome size occurred in cultivars of *P. communis*, the lowest in *P.* ×*myloslavensis* F_1. In this study interspecific nuclear DNA content variation ($p<0.05$) was observed for species *P. communis* and the hybrid, whereas no intraspecific variation was detected for *P. communis* cultivars (Table 4). 2C value for 'Conference' cultivar was smaller than other three cultivar of *P. communis* and was close to DNA content of *P. salicifolia* species. The determined genome size for all genotypes studied, ranged from 1086 Mbp to 1118 Mbp. The variation between the lowest and highest pears was only 2.54 % of mean diploid DNA content. The average genome size of the hybrid was smaller than putative parents, *P. communis* 'Conference' and *P. salicifolia*.

Discussion and conclusions

The previous study on basic characteristics of *P.* ×*myloslavensis* clearly indicated its hybrid origin (Antkowiak et al., 2008). In most cases a newly discovered, single individual of this species had features that were intermediate between the parental species, i.e. *P. salicifolia* and *P. communis*. According to the leaf morphology it showed a greater similarity to *P. salicifolia*, but in respect of fruit morphology it was more similar to the *P. communis*. Likewise, current research also confirmed hybrid origin of *P.* ×*myloslavensis*. Especially, pollen grains had most features similar to both parental species. In respect to epidermal micromorphology, seeds of *P.* ×*myloslaviensis* were more like *P. salicifolia* than *P. communis*. In contrast, anatomical structure of the hybrid seed coat was more consistent with *P. communis* than *P. salicifolia*.

According to the date obtained by Wojciechowski and Antkowiak (2009), two cultivars of *P. communis* used in this experiment ('Conference' and 'Corola') are self-incompatible, but are fully compatible with the wild species *P. pyraster*, when independently crossed as maternal parent. The data obtained from the experiment presented in this paper show that the new pear species, *P.* ×*myloslavensis* is self-incompatible and cross-compatible only with one of four tested *P. communis* cultivars ('Conference') and with *P. salicifolia*. Quite interesting results were obtained from the reciprocal crosses of *P. communis* 'Conference' and *P. salicifolia*. In this case unilateral cross-compatibility was observed only in this cross combination where *P. salicifiolia* was used as the maternal form.

Present study revealed that average genome size of *P.* ×*myloslavensis* was smaller than putative parents, *P. communis* 'Conference' and *P. salicifolia*. This may be caused by the elimination of chromosomal fragments or selected DNA sequences after hybridization. Data on genome size in *Pyrus* genus are scarce. Only few reports on members of the *Pyrus* are published. For *P. communis* reported by Arumuganathan and Earle (1991) the determined 2C DNA content was 1.11 pg and was in the same range as our results. Dickson et al. (1992) and Jędrzejczyk and Śliwińska (2010) published C-values for *P. calleryana* and *P. elaegrifolia*. The genome size for these species was 1.26 pg/2C and 1.15 pg/2C, respectively. According to Soltis et al. (2003) *Pyrus* genus would be categorized as a group of plants with 'very small' genomes. To the best of our knowledge this is the first report on the estimation of nuclear DNA content of *P. salicifolia*, and the hybrid *P.* ×*myloslavensis* as well as of the four cultivars of *P. communis*. The information about the genome size will be reported to the RBG Kew DNA C-values base.

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