Acta Societatis Botanicorum Poloniae
Journal homepage: pbsociety.org.pl/journals/index.php/asbp
ORIGINAL RESEARCH PAPER Received: 2013.01.23 Accepted: 2013.05.23 Published electronically: 2013.06.21 Acta Soc Bot Pol 82(2):147–156 DOI: 10.5586/asbp.2013.016

Malus ×oxysepala (*M. domestica* Borkh. × *M. sylvestris* Mill.) – a new spontaneous apple hybrid

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Abstract

A study of the three *Malus* species (*M. domestica*, *M. sylvestris*, and a hybrid species, *M. domestica* × *M. sylvestris*, which was named *M. ×oxysepala*) was carried out based on the morphological and micromorphological features and molecular investigation. Observations performed for 47 quantitative traits showed that this hybrid species exhibits intermediate values between *M. domestica* and *M. sylvestris*, or are similar to traits of one of the parents. Sepals proved to be the best diagnostic feature because they were acuminate and much longer than sepals in *M. domestica* and *M. sylvestris*. Seed testa cells are distinct, rimmed with straight anticlinal walls and strongly bulged periclinal walls. Simultaneous genetic analyses based on PCR RAPD reactions fully confirmed earlier morphological observations. Genetic profiles of the hybrid obtained with the use of 30 primers, next to species-specific amplification products, contain common products with each of the parents. However, both the profile analysis and the dendrogram constructed on its basis showed that the hybrid is genetically closer to *M. sylvestris*.

Keywords: *Malus domestica*, *Malus sylvestris*, *Malus* hybrid, taxonomy, SEM, PCR RAPD, genetic similarity

Introduction

We know 25–47 species from the genus *Malus* in Europe, Asia and North America [1,2]. In Poland one species is found in the wild, i.e. *Malus sylvestris*, and one escaped species is disseminating mainly along transportation routes, i.e. *Malus domestica*. The latter species in the temperate climate zone, both in the northern and southern hemispheres, is the most commonly cultivated fruit tree. Numerous species, and even more numerous cultivars, are grown for ornamental purposes in parks, in housing district green areas, and in large city green areas. In Poland, these include the following taxa: *Malus baccata*, *M. floribunda*, *M. pumila*, *M. ×purpurea*, *M. ×scheideckeri*, and *M. sieversii* [3].

There are no genetic barriers between *M. sylvestris* and *M. domestica* [4]. Although literature sources in the description of *M. sylvestris* state that, at present, it is hardly ever found in the pure form because it frequently crosses with cultivars [1,5–7], a description of the hybrid species is nowhere to be found [8–16].

The aim of this study was to indicate diagnostic traits for the hybrid between *M. sylvestris* and *M. domestica*. In view of the variability in plants caused by environmental factors, additional molecular analyses (PCR RAPD) were conducted verifying the position of the described species in relation to *M. sylvestris* and *M. domestica*. This method is used successfully in analyses concerning different aspects of the genus *Malus* [17–19].

Material and methods

In the course of floristic studies in the Wielkopolska region, four localities of *M. domestica* × *M. sylvestris* were found. Traits, on the basis of which the encountered hybrid was identified, included the ratio of the length to the width of sepals within the range of 2–3.5 and sharply pointed sepals, small flowers comparable to *M. sylvestris*, leaves on long shoots were similar in size and shape to those in *M. domestica*, while the smallest fruits were largest in *M. sylvestris* and the largest were smallest in *M. domestica*.

Due to the limited material from *M. sylvestris* (only one fruit-bearing tree was available) analyses were conducted on one tree from each analyzed taxon (Fig. 1): *M. sylvestris*: Ignacewo (N: 52°15′57″, E: 19°04′26″), the Chodów commune, Wielkopolska province – an old, no longer used evangelical cemetery. *M. ×oxysepala*: Ługi (N: 51°58′47″, E: 17°11′32″), the Książ Wielkopolski commune, Wielkopolska province – on the roadside next to a forest. *M. domestica*: Książ Wielkopolski (N: 52°03′28″, E: 17°13′50″), the Książ Wielkopolski commune, Wielkopolska province – in an avenue at a road to a no longer used railway station.

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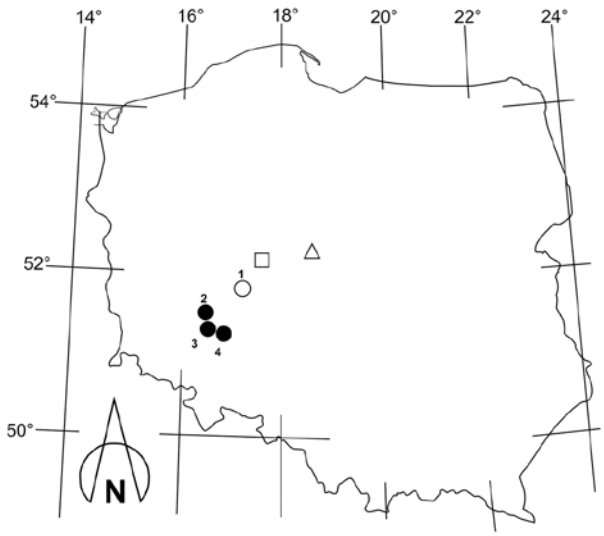

Fig. 1 Locations of all the recorded localities of *Malus* ×*oxysepala* in Poland: 1 – Ługi, the Książ Wielkopolski commune (analyzed population – an empty circle); 2 – Dębowa Łęka, the Wschowa commune; 3 – Gołkowice, the Pęcław commune; 4 – Leszkowice, the Pęcław commune (non-analyzed populations – a black circle) and *Malus sylvestris* – Ignacego, the Chodów commune (analyzed population – an empty triangle) and *Malus domestica* Książ Wielkopolski, the Książ Wielkopolski commune (analyzed population – an empty rectangle).

Analysis of quantitative and qualitative morphological traits

Comparative observations of morphology and micromorphology of the analyzed species were conducted on leaves, flowers, fruits and seeds. Flowers were collected at the beginning of May and fruits towards the end of September. A total of 47 quantitative traits and 14 qualitative traits were analyzed. Measurements were taken using a ruler, calipers and a PZO type 131 stereoscopic microscope. A total of 30 measurements of each trait were taken for each species.

Analyzed quantitative traits included:

(***i***) long and short shoot leaves: length of leaf petiole, length of leaf blade, width of leaf blade, length of blade top, width of blade top, number of lateral veins, angle between main vein and second lateral vein, length of leaf blade/width of leaf blade, length of leaf blade/length of leaf petiole, length of leaf blade/No. of lateral veins, width of leaf blade/width of blade top;

(***ii***) flowers: length of petals, width of petals, length of sepals, width of sepals, length of petals/width of petals, length of sepals/width of sepals, length of petals/length of sepals;

(***iii***) fruits and seeds: length of fruit pedicle, fruit diameter, fruit height, length of fruit ventricle, width of fruit ventricle, number of seeds, length of seed, width of seed, seed thickness, length of seed hilum, width of seed hilum, distance between the widest part of seed and hilum, fruit diameter/fruit height, fruit height/length of fruit pedicle, length of seed/width of seed, seed thickness/width of seed, length of seed hilum/width of seed hilum, length of seed/distance between the most wide part of seed and hilum.

The significance of differences between species in terms of all quantitative traits mentioned above was verified using one-way ANOVA. Tests were performed separately for each of the analyzed traits. If differences were found in a given trait, Tukey's post-hoc test was used to determine which species differ and which are identical in relation to that trait. The analysis of the discriminatory function was applied to definitely identify which traits play a key role in the discrimination of *Malus* ×*oxysepala*. In accordance with the requirements of the test, variables resulting from the ratio of two different variables (e.g. length of leaf blade/width of leaf blade) were omitted; thus, 30 traits were tested. All the analyses were performed using Statistica software (Statsoft ver. 9).

The following qualitative morphological traits were described:

(***i***) long and short shoot leaves: shape of leaf blade, type of leaf tip, pubescence of leaf blade underside;

(***ii***) flowers: shape of corolla petals, margin of petals, apex of calyx sepals;

(***iii***) fruits and seeds: shape of fruits, type of fruit apex, type of fruit base, shape of seeds, seed sculpture.

Photographs of the seed sculpture surface under a scanning electron microscope (SEM) were obtained with a Zeiss Evo'40 scanning electron microscope at an accelerating voltage of 15 kV and the obtained images were analyzed.

Morphological characteristics were used to create a key for determination of studied species. Three specimen of the hybrid, that were not studied in detail, were used in control studies, i.e. they were determined on the basis of the developed key.

Molecular analyses

Total genomic DNA was extracted from fresh and young leaves of individual plants, using a cetyl trimethylammonium bromide (CTAB) method [20] with minor modifications. Young leaves were ground to a powder in liquid nitrogen by using a mortar and pestle. The powdered tissue was mixed with 1–2 ml extraction CTAB buffer with addition of 3% PVP (polyvinylpyrrolidone) and 0.4% β-mercaptoethanol. The mixture was then incubated at 65°C for 45 min. Next, DNA was extracted using a mixture of chloroform-octanol (octyl alcohol; 24:1). After centrifugation, DNA was recovered from the aqueous phase through precipitation with 96% ethanol, washed with 70% ethanol, air-dried and resuspended in sterile water. The DNA concentration was estimated by spectrophotometer, adjusted to 20 ng μl^{-1} and used as a template in PCR reactions.

PCR RAPD amplification was performed in volumes of 16 µl containing a ready-to-use PCR Master Mix (2×; Fermentas), 20 pM 10-mer primer and 50 ng of template DNA. For identification of RAPD markers unique for species studied, thirty 10-nucleotide primers were selected from commercially available primer kits H and L (Operon Technologies Inc., USA). DNA amplification reactions were performed in a thermocycler (M. J. Research, Inc.) programmed for 40 cycles divided into two stages differing in terms of annealing temperature. The first 20 cycles were preceded by initial denaturation at 95°C for 300 s, each cycle was composed of denaturation step at 92°C for 90 s, annealing step at 35°C for 90 s and extension step at 72°C for 120 s, after which in order to enhance specificity, temperature of annealing was increased to 38°C and another 20 successive cycles followed, in which the other stages remained unchanged. The reaction was completed by final synthesis at 72°C for 300 s and storage at 4°C until turned off.

The amplified products were separated by electrophoresis in 1.5% agarose gels in 1× TBE buffer, containing ethidium bromide, in the presence of size markers. DNA bands were photographed under ultra violet light, utilizing the photo documentation system, GBOX (Syngen Biotech). Selected reactions were also separated in 6% polyacrylamide gels and the results were visualized by silver staining.

Tab. 1 Mean values ±*SD* of investigated features of *Malus sylvestris*, *M.* ×*oxysepala* and *M. domestica*.

Features of plants	Results (mm; mean ±*SD*; min–max)			ANOVA	Tukey HSD		
	M. sylvestris	*M.* ×*oxysepala*	*M. domestica*	*P*	*M. sylvestris*	*M.* ×*oxysepala*	*M. domestica*
Long shoot leaves							
Length of leaf petiole	24.50 ±3.24 15–31	33.67 ±6.85 22–47	25.43 ±1.62 3–45	0.000027	b	a	b
Length of leaf blade	62.33 ±11.34 38–79	75.67 ±23.69 8–105	89.03 ±15.31 65–117	0.000000	c	b	a
Width of leaf blade	40.27 ±6.64 22–51	52.37 ±10.78 28–72	46.43 ±7.91 31–60	0.000003	c	a	b
Length of blade top	5.67 ±2.40 1–10	7.87 ±2.96 1–12	7.87 ±1.83 4–12	0.000577	b	a	a
Width of blade top	5.13 ±1.50 1–8	7.00 ±2.18 2–11	6.07 ±0.78 5–7	0.00009	b	a	ab
No. of lateral veins	6.27 ±1.53 4–10	7.03 ±1.33 5–12	7.00 ±1.51 5–10	NS	-	-	-
Angle between main vein and second lateral vein	25.70 ±6.33 13–41	27.87 ±6.43 15–40	25.10 ±5.84 13–37	NS	-	-	-
Length of leaf blade/width of leaf blade	1.6 ±0.23 1.08–2	1.43 ±0.28 0.15–1.78	1.9 ±0.25 1.38–2.3	0.000000	b	b	a
Length of leaf blade/length of leaf petiole	2.5 ±0.36 1.82–3.2	2.26 ±0.59 0.22–3	7.2 ±9.5 1.97–31.7	0.000981	b	b	a
Length of leaf blade/No. of lateral veins	10.2 ±1.76 6.33–15	10.87 ±3.47 1.33–16.33	13 ±2.23 9.5–17.8	0.000180	b	b	a
Width of leaf blade/width of blade top	8.9 ±4.56 4.33–30	8.18 ±3.03 4.91–21	7.8 ±1.74 5.5–11.6	NS	-	-	-
Short shoot leaves							
Length of leaf petiole	27.53 ±6.19 13–37	29.57 ±9.66 10–47	34.70 ±6.24 25–47	0.001278	b	b	a
Length of leaf blade	57.57 ±11.52 25–77	60.77 ±15.49 27–88	85.23 ±22.93 40–118	0.000000	b	b	a
Width of leaf blade	36.20 ±5.59 22–46	37.80 ±8.38 20–53	45.50 ±5.86 33–55	0.000001	b	b	a
Length of blade top	4.67 ±1.92 1–9	6.60 ±2.42 2–11	6.60 ±3.00 0–11	0.003452	b	a	a
Width of blade top	4.83 ±1.49 2–8	5.00 ±1.39 2–7	5.07 ±1.84 0–7	NS	-	-	-
No. of lateral veins	6.37 ±0.93 4–8	6.03 ±1.16 4–9	7.00 ±2.17 4–13	0.047888	ab	b	a
Angle between main vein and second lateral vein	24.27 ±5.87 12–32	21.27 ±3.80 10–27	25.23 ±5.10 17–37	0.007743	ab	b	a
Length of leaf blade/width of leaf blade	1.59 ±0.22 1.14–2	1.60 ±0.18 1.30–2.07	1.9 ±0.42 1–2.8	0.000479	b	b	a
Length of leaf blade/length of leaf petiole	2.13 ±0.34 1.49–3.23	2.14 ±0.35 1.46–2.79	2.4 ±0.44 1.56–3.3	0.002498	b	b	a
Length of leaf blade/No. of lateral veins	9.12 ±1.91 5.83–14	10.08 ±2.00 6.75–14.6	12.6 ±3.39 6.67–20	0.000002	b	b	a
Width of leaf blade/width of blade top	8.17 ±2.86 4.14–18.5	7.90 ±1.91 5.50–13.5	9.1 ±3.11 6.14–20	NS	-	-	-
Flowers							
Length of petals	14.70 ±1.53 11–18	14.53 ±2.74 10–19	22.47 ±3.22 15–29	0.000000	b	b	a
Width of petals	8.63 ±1.22 6–12	9.30 ±2.07 6–14	15.50 ±2.42 10–22	0.000000	b	b	a

Tab. 1 *(continued)*

Features of plants	Results (mm; mean ±*SD*; min–max)			ANOVA	Tukey HSD		
	M. sylvestris	*M.* ×*oxysepala*	*M. domestica*	*P*	*M. sylvestris*	*M.* ×*oxysepala*	*M. domestica*
Length of sepals	5.10 ±0.76 3–6	8.73 ±0.78 7–10	7.83 ±0.83 6–10	0.000000	c	a	b
Width of sepals	2.87 ±0.43 2–4	3.53 ±0.57 2–4	4.37 ±0.56 3–5	0.000000	c	b	a
Length of petals/width of petals	1.72 ±0.18 1.4–2.14	1.58 ±0.17 1.29–1.88	1.46 ±0.14 1.18–1.93	0.000000	a	b	c
Length of sepals/width of sepals	1.80 ±0.26 1–2.5	2.52 ±0.36 2–3.50	1.81 ±0.23 1.4–2.33	0.000000	b	a	b
Length of petals/length of sepals	2.95 ±0.59 1.83–5	1.67 ±0.28 1.2–2.11	2.9 ±0.54 2–4.14	0.000000	a	b	a
Fruits and seeds							
Length of fruit pedicle	14.87 ±3.26 9–20	10.70 ±3.13 4–16	12.07 ±3.51 7–21	0.000018	a	b	b
Fruit diameter	23.60 ±3.51 17–33	39.60 ± 6.54 29–50	56.40 ±6.09 46–72	0.000000	c	b	a
Fruit height	18.77 ±2.14 14–26	35.47 ±4.80 26–43	45.90 ±7.97 38–72	0.000000	c	b	a
Length of fruit ventricle	7.57 ±1.10 6–11	9.70 ±1.32 7–13	12.97 ±2.98 1–21	0.000000	c	b	a
Width of fruit ventricle	5.63 ±1.45 3–11	7.07 ±1.20 5–9	9.07 ±1.64 6–13	0.000000	c	b	a
No. of seeds	4.73 ±2.30 1–9	5.53 ±3.14 0–10	3.37 ±1.13 1–5	0.002186	ab	b	a
Length of seed	4.63 ±0.35 4.06–5.31	6.39 ±0.62 5.3–7.4	7.15 ±0.44 5.7–8.2	0.000000	c	b	a
Width of seed	3.06 ±0.41 2.37–4	4.15 ±0.27 3.8–5	4.68 ±0.33 4–5.7	0.000000	c	b	a
Seed thickness	1.39 ±0.19 0.94–1.87	2.93 ±0.17 2.5–3	2.43 ±0.37 1.5–3	0.000000	c	a	b
Length of seed hilum	0.84 ±0.14 0.55–1.06	1.39 ±0.31 1–2.2	1.62 ±2.34 0.8–12	NS	-	-	-
Width of seed hilum	0.37 ±0.05 0.31–0.5	0.59 ±0.08 0.5–0.8	0.55 ±0.09 0.4–0.7	0.000000	b	a	a
Distance between the most wide part of seed and hilum	2.86 ±0.28 2.37–3.56	4.14 ±0.57 2.8–5.4	4.66 ±0.48 3.5–5.5	0.000000	c	b	a
Fruit diameter/fruit height	1.26 ±0.14 1–1.83	1.11 ±0.09 0.95–1.27	1.24 ±0.11 0.91–1.41	0.000005	a	b	a
Fruit height/length of fruit pedicle	1.33 ±0.35 0.8–2.11	3.66 ±1.35 1.94–7.75	4.11 ±1.43 2.33–8	0.000000	b	a	a
Length of seed/width of seed	1.53 ±0.19 1.28–1.98	1.54 ±0.18 1.25–1.83	1.53 ±0.14 1.21–1.9	NS	-	-	-
Seed thickness/width of seed	0.46 ±0.05 0.29–0.53	0.71 ±0.06 0.60–0.79	0.52 ±0.09 0.35–0.7	0.000000	c	a	b
Length of seed hilum/width of seed hilum	2.29 ±0.45 1.1–3.23	2.40 ±0.66 1.43–4.4	3.11 ±4.73 1.14–24	NS	-	-	-
Length of seed/distance between the most wide part of seed and hilum	1.63 ±0.10 1.49–1.88	1.56 ±0.18 1.23–2.04	1.55 ±0.12 1.35–1.8	NS	-	-	-

ANOVA were performed separately for each of analyzed feature. The letters in rows indicate significant differences with *P*-level < 0.05 according to Tukey's HSD test; dashes mean absence of differences according to Tukey's test. NS – absence of significant differences between analyzed species according to ANOVA.

Obtained genetic profiles (fingerprints) were analyzed in terms of polymorphic RAPD markers unique for individual species and common for one of the assumed parents and the hybrid. The percentage of polymorphic products indicating inheritance of traits from each was calculated. All profiles were compared by calculation according to Nei and Li [21]. Each polymorphic band was treated as a unit character and scored as binary codes (0/ 1). A similarity matrix was constructed from binary data using formula $NL = 2a/(b + c)$ where a is the number of RAPD fragments shared by the two individuals and b and c are the number of RAPD fragments in each. The genetic distance ($D = 1 - NL$) and the UPGMA program were used for cluster analysis and generation of the dendrogram, graphically determining interrelations between the analyzed taxa. The dendrogram was created using Statistica software (Statsoft ver. 9).

Results

Analysis of quantitative and qualitative morphological traits

QUANTITATIVE TRAITS. *M.* ×*oxysepala* differ significantly from parental plants in terms of eight analyzed traits: length of long shoot leaf petiole, width of long shoot leaf blade, length of sepals, length of sepals/width of sepals, length of petals/length of sepals, seed thickness, fruit diameter/fruit height and seed thickness/width of seed (Tab. 1). *M.* ×*oxysepala* could be distinguished by wider leaf blades (mean 52 mm) and longer petioles (mean 34 mm) on long shoot leaves as compared to both parental species (Fig. 2a–c). Sepals (mean 8.7 mm) are much longer than those in *M. domestica* and *M. sylvestris* (Fig. 3a–c). Similarly, the length of sepals to width of sepals ratio (mean 2.5) is significantly higher. Fruit diameter to fruit height ratio (mean 1.1) is lower, whereas seeds (mean 2.9 mm) are thicker than analogous traits in parental species (Fig. 3g–l).

Fourteen traits make the hybrid resemble *M. sylvestris*: length of long shoot leaf blade/width of leaf blade, length of long shoot leaf blade/length of leaf petiole, length of long shoot leaf blade/No. of lateral veins, length of short shoot leaf petiole, length of short shoot leaf blade, width of short shoot leaf blade, length of short shoot leaf blade/width of leaf blade, length of short shoot leaf blade/length of leaf petiole, length short shoot of leaf blade/No. of lateral veins, No. of lateral veins on short shoot leaf, angle between main vein and second lateral vein on short shoot leaf, length of petals, width of petals, No. of seeds (Tab. 1). The common effect of traits that concern the long shoot leaves causes that leaves of *M.* ×*oxysepala* and *M. sylvestris* look to be wide-elliptic (Fig. 2a–c). Leaves on short shoots have smaller but relatively wider blades (mean length 61 mm for *M.* ×*oxysepala* and 58 mm for *M. sylvestris*; mean width 38 mm and 36 mm, respectively; length of leaf blade to width of leaf blade ratio 1.6 for both species) with shorter petioles (mean 30 mm and 28 mm respectively) as compared to *M. domestica*. Flowers of *M. sylvestris* and *M.* ×*oxysepala* are characterized by smaller petals (mean length 14.5 mm and 14.7 mm, respectively, mean width 9.3 mm and 8.6 mm, respectively; Fig. 3d–f). Unlike *M. domestica*, fruits could contain more than five seeds.

Six traits make *M.* ×*oxysepala* similar to *M. domestica*: length of long shoot leave blade top, width of long shoot leave blade top, length of short shoot leave blade top, length of fruit pedicle, width of seed hilum and fruit height/length of fruit pedicle (Tab. 1). Both species are characterized by relatively large blade tops on long shoot leaves and short shoot leaves (long shoots: mean length 7.9 mm for both species, mean width 7 mm for *M.* ×*oxysepala* and 6.1 mm, for *M. domestica*; short shoots: mean length 6.6 mm for both species). Fruits' pedicles (mean 10.7 mm for *M.* ×*oxysepala* and 12.1 mm for *M. domestica*) are shorter than those observed in *M. sylvestris*, and the ratio of fruit height to length of fruit pedicle (mean 3.7 and 4.1, respectively) is higher than in *M. sylvestris* (Fig. 3g–i). Seed hilum is relatively wide (mean 0.6 mm and 0.5 mm, respectively).

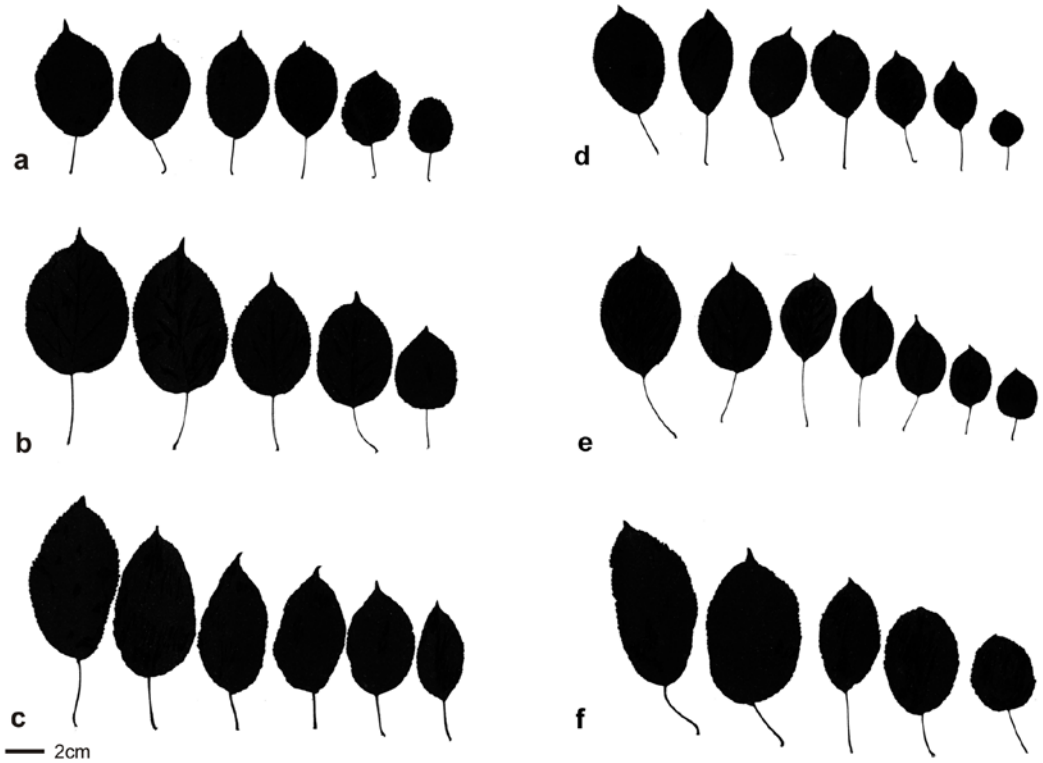

Fig. 2 Leaves from long shoots (**a–c**) and short shoots (**d–f**). **a,d** *Malus sylvestris*. **b,e** *M.* ×*oxysepala*. **c,f** *M. domestica*.

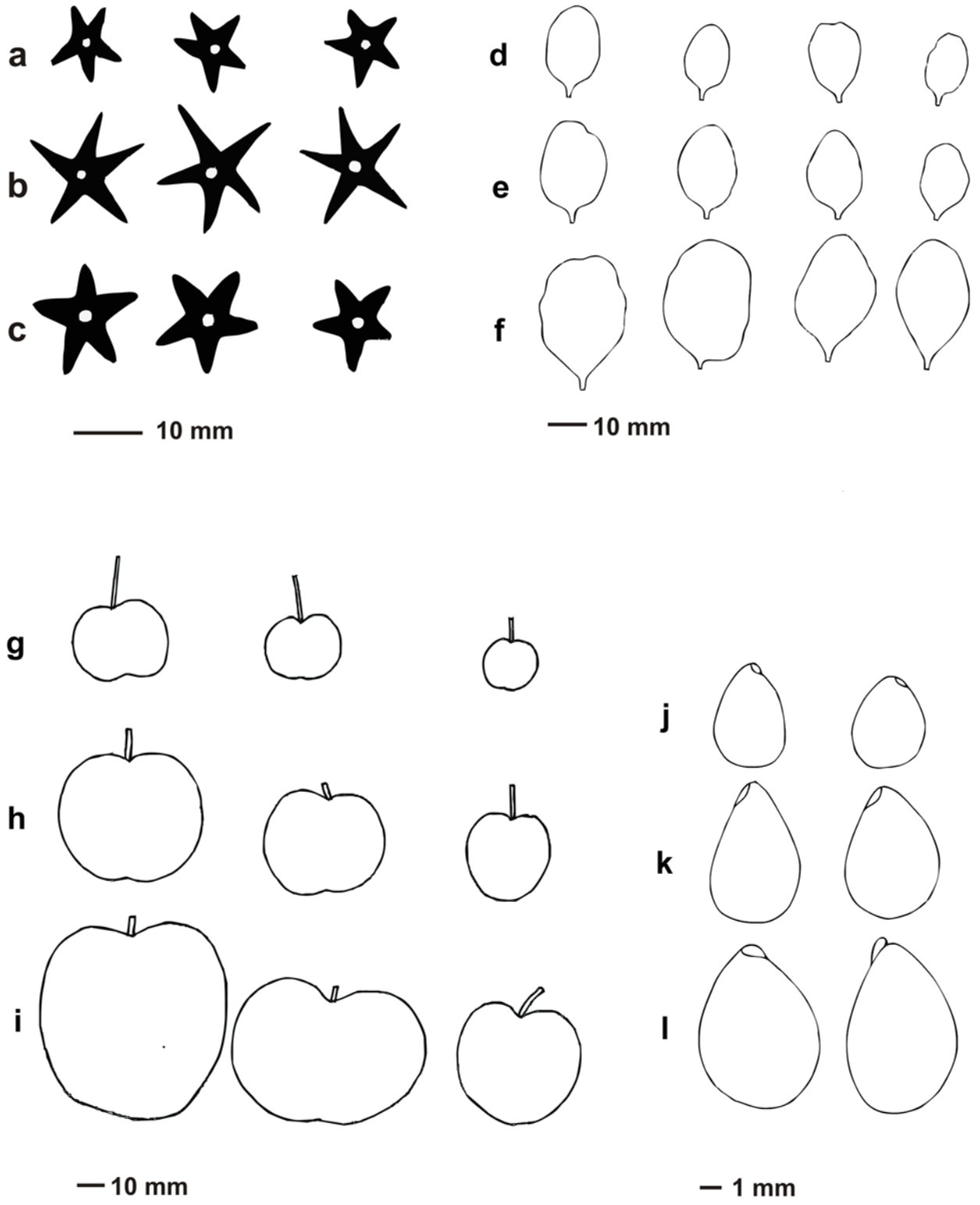

Fig. 3 Sets of taxonomic traits: sepals (**a–c**), petals (**d–f**), fruits (**g–i**), seeds (**j–l**). **a,d,g,j** *Malus sylvestris*. **b,e,h,k** *M.* ×*oxysepala*. **c,f,i,l** *M. domestica*.

Ten traits in the hybrid species take intermediate values between those of both parents: length of long shoot leaf blade (mean 75.7 mm), width of sepals (mean 3.5 mm), length of petals to width of petals ratio (mean 1.6), fruit diameter (mean 40 mm), fruit height (mean 35 mm), length of fruit ventricle (mean 9.7 mm), width of fruit ventricle (mean 7.1 mm), length of seed (mean 6.4 mm), width of seed (mean 4.2 mm), distance between the most wide part of seed and hilum (mean 4.1 mm).

M. domestica, *M.* ×*oxysepala* and *M. sylvestris* do not differ significantly in terms of nine traits: No. of lateral veins on long shoot leaf, angle between main vein and second lateral vein on long shoot leaf, width of long shoot leaf blade/width of blade top, width of short shoot blade top, width of short shoot leaf blade/width of blade top, length of seed hilum, length of seed/width of seed, length of seed hilum/width of seed hilum, length of seed/distance between the most wide part of seed and hilum.

Results of the discriminant analysis are presented in Fig. 4. Discrimination of the analyzed species is highly significant (Wilks's Lambda = 0.00172, approx. F (60.16) = 44.751, $P < 0.000$). The chi-square test of discriminant analyses indicates that both functions are statistically significant. The first function explains 79% variance, while the second function explains 21% variation. The first discriminant function is determined the strongest by the negative coefficients for fruit diameter and positive coefficients for the number of seeds (Fig. 5). With reference to this traits *M. domestica* and *M. sylvestris* differ the most, but *M.* ×*oxysepala* is between. The second function clearly discriminates *M.* ×*oxysepala* from *M. sylvestris* and *M. domestica*. The main traits discriminating the hybrid from the parents are length of sepals and length and width of long shoot leaves.

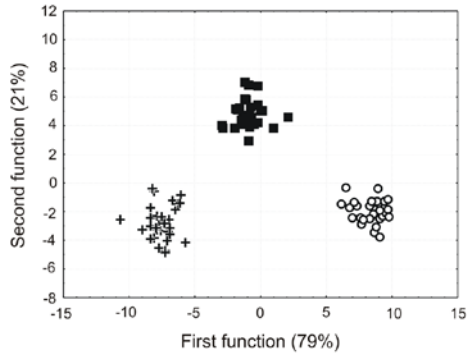

Fig. 4 The scatter graph of the analyzed traits' canonical values in *Malus domestica* (crosses), *M.* ×*oxysepala* (squares) and *M. sylvestris* (circles).

QUALITATIVE TRAITS. *M.* ×*oxysepala*, *M. sylvestris* and *M. domestica* exhibit differences or are identical in terms of the analyzed traits, or intermediate values between *M. domestica* and *M. sylvestris* are found in the hybrid. In all the species leaves on long shoots are elliptic (or wide-elliptic) with an acute tip, while on short shoots they are most frequently elliptic, but also obovate or orbicular with either an acute or obtuse tip (Fig. 2). Pubescence on the underside of leaves in *M.* ×*oxysepala* is scarce and intermediate between those of the parents (Fig. 6). Petals in all the species are elliptic with a slightly undulated margin (Fig. 3d–f). In turn, sepals in *M. sylvetris* and *M. domestica* acute, while in *M.* ×*oxysepala* acuminate (Fig. 3a–c). In the observed species, fruits are most frequently round obcordate at the tip and the base (Fig. 3g–i), while seeds are ovate (Fig. 3j–l). Seed surface sculpture shows differently bulged periclinal walls that are flat in *M. domestica*, bulged in *M. sylvetris* and strongly bulged in *M.* ×*oxysepala*. Anticlinal walls are always straight (Fig. 7).

Molecular analyses

Thirty primers were selected for the analyses of phylogenetic linkages among parental species *M. domestica*, *M. sylvestris* and the hybrid *M.* ×*oxysepala*. Except for four (only monomorphic products were obtained), all the others provided diverse band profiles, distinctly indicating a hybrid character of *M.* ×*oxysepala*. A total of 249 products were obtained of which 150 (60.2%) were composed of polymorphic bands. No significant differences were observed in the numbers of amplified products in individual species (168 products in case of *M. domestica* and 172 each in the others). Differences were observed, rather, in their distribution, making it possible to state the presence of both bands unique for each taxon and common for the hybrid and each of the parents (Fig. 8). In the case of *M.* ×*oxysepala* among 172 products, 52 comprised bands found in one of the

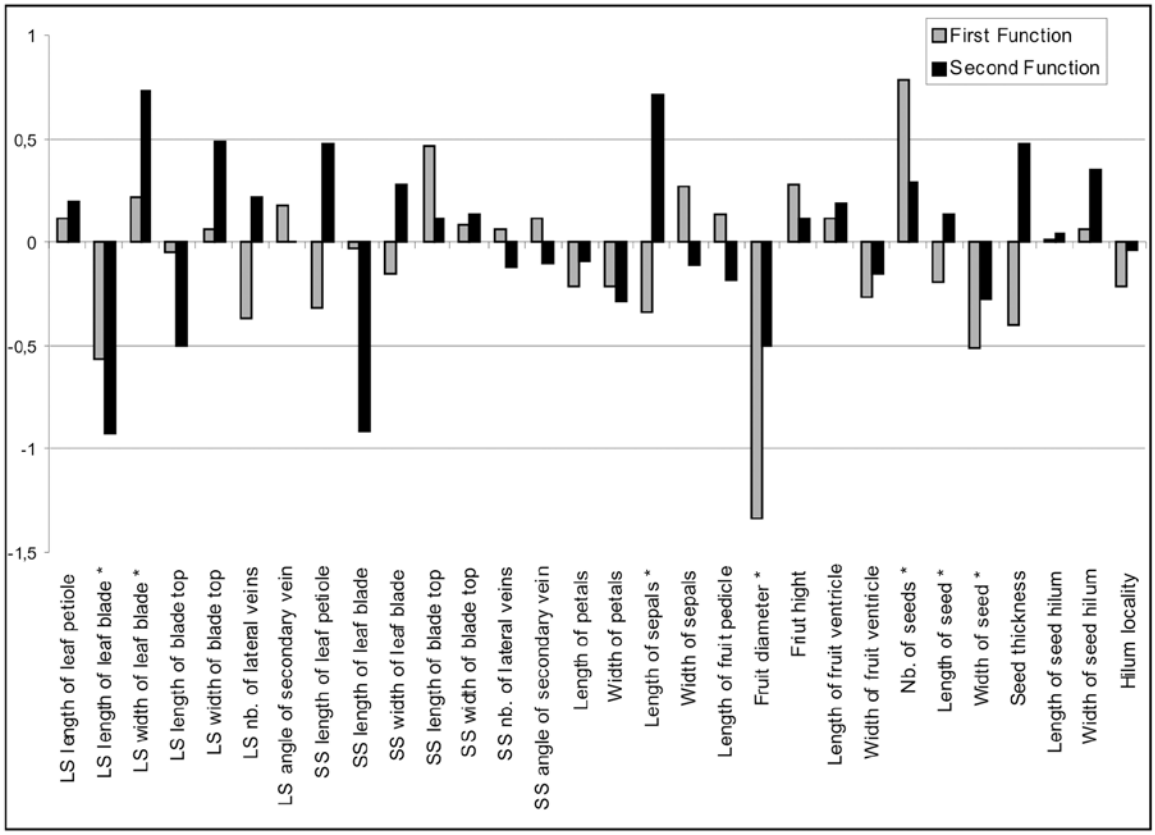

Fig. 5 Coefficients standardized for canonical variables of analyzed traits of *Malus sylvestris*, *M.* ×*oxysepala* and *M. domestica*. The asterisks denote traits significantly influencing the model. LS – long shoot; SS – short shoot.

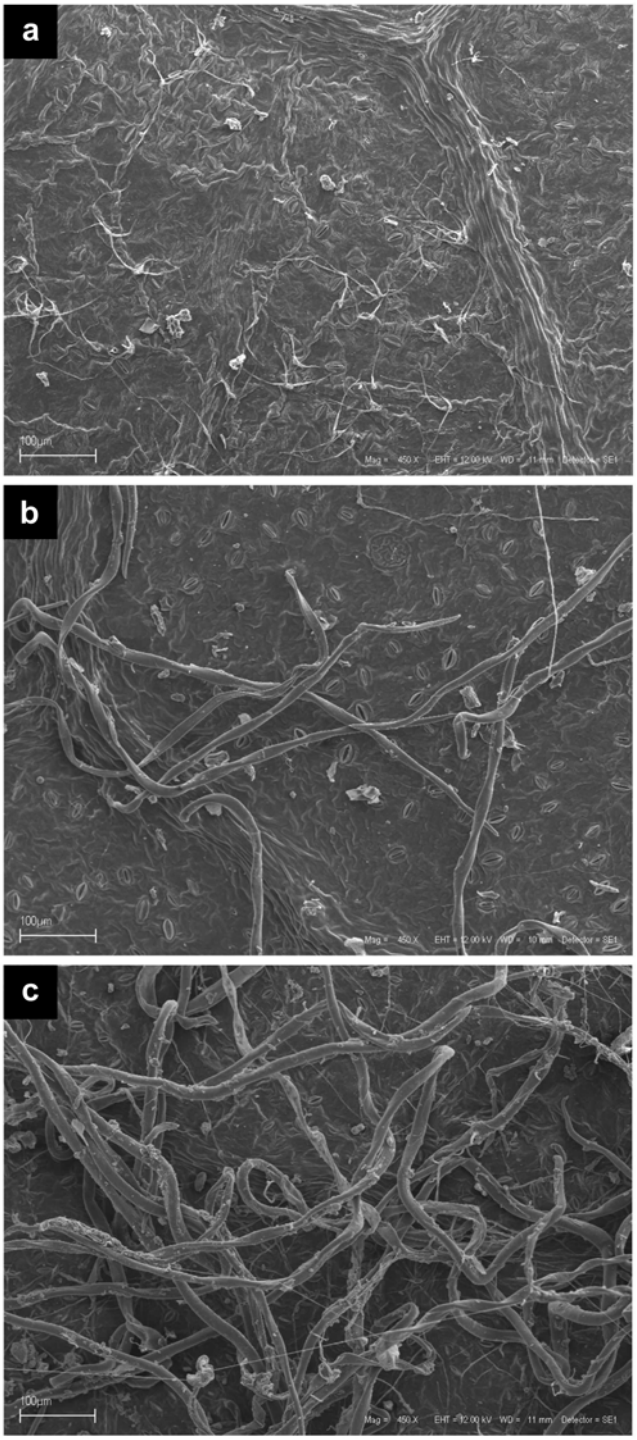

Fig. 6 Pubescence on leaf underside on long shoot between the first and second vein. **a** *Malus sylvestris*. **b** *M.* ×*oxysepala*. **c** *M. domestica*.

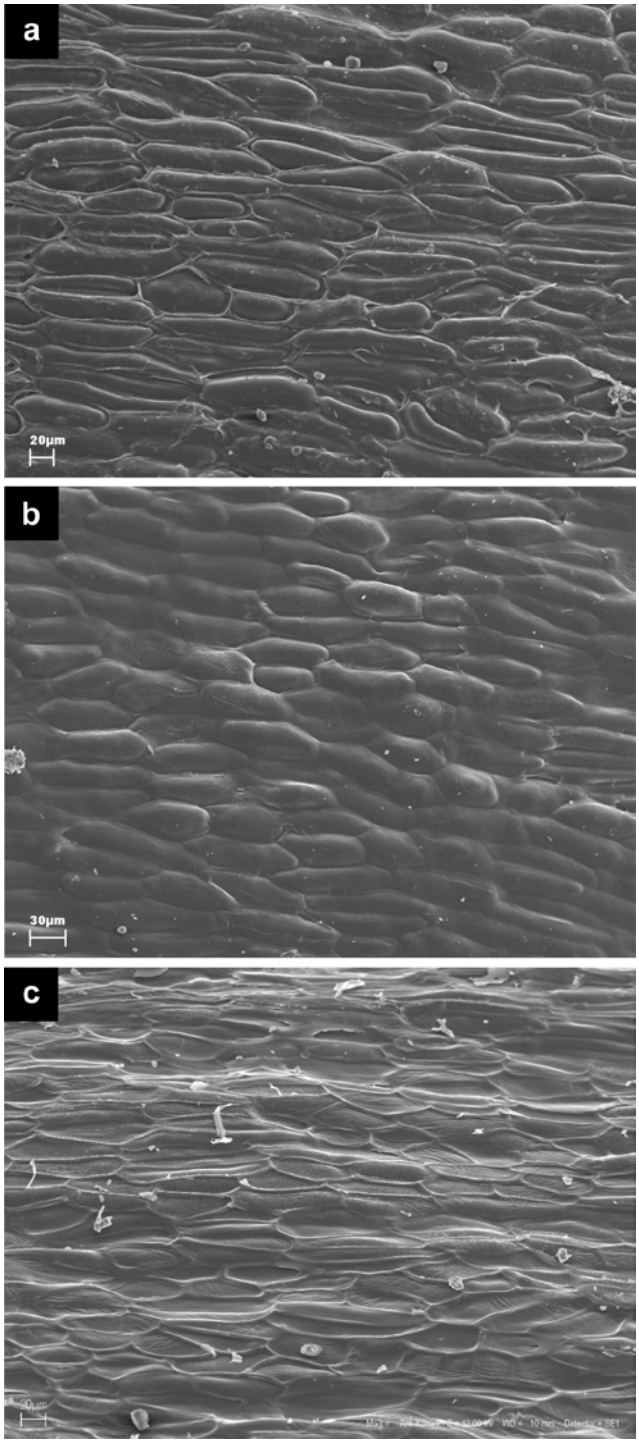

Fig. 7 Seed surface sculpture. **a** *Malus sylvestris*. **b** *M.* ×*oxysepala*. **c** *M. domestica*.

parents, i.e. 30 (17.4%), common with *M. sylvestris*, and 22 (12.8%) with *M. domestica*. At the same time, the presence of 17 (6.8%) products unique for the hybrid was found. Thus, the analysis confirmed the observed phenotypic traits for individual taxa, indicating the hybrid status of the described species. From data, we may also infer a certain advantage of traits from *M. sylvestris*.

In order to verify the phylogenetic relationships among the analyzed taxa, all the polymorphic products were counted, and the percentage of the common products was compared. On the basis of the created datasheet, a matrix was established for the genetic distance and a dendrogram was plotted (Fig. 9). Analysis of the dendrogram confirms previous assumptions that *M.* ×*oxysepala* is genetically closer to *M. sylvestris* than to *M. domestica*.

Diagnosis

Malus ×*oxysepala*, A. Czarna, sp. hybr. nova.

Malus ×*oxysepala, verosimiliter inter M. sylvestris et M. domestica hybrida, ab illa species foliis adultis parce pubescentibus differt, ab altera petalis brevibus (ad 20 mm longis), pomum ad 50 mm diametro, foliis minus pubescentibus et seminibus sculptura discrepat, ab ambobus parentibus sepalis longioribus dignoscitur.*

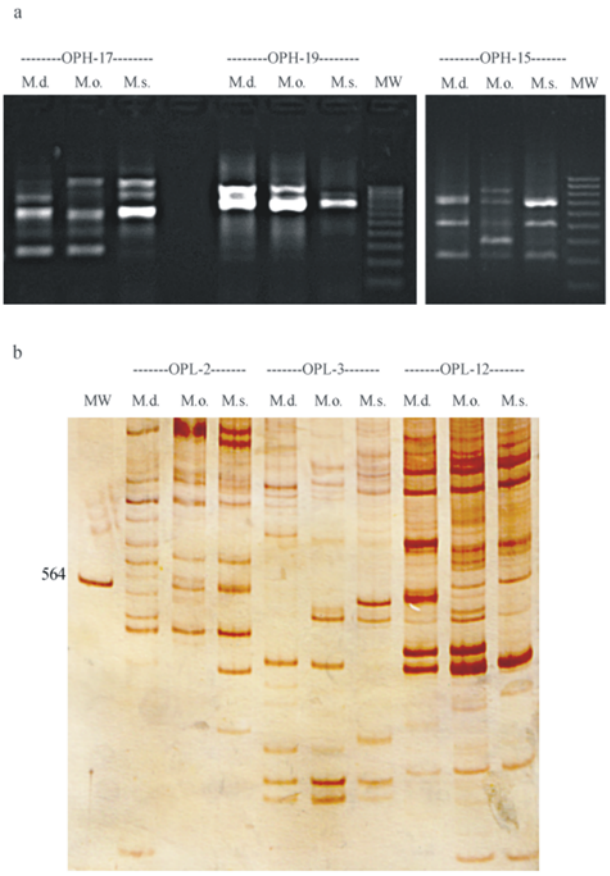

Fig. 8 Examples of different RAPD fingerprints of *Malus* specimens generated by oligonucleotide primers indicated on top. Letters M.d., M.o. and M.s. refer to *Malus domestica*, *M.* ×*oxysepala* and *M. sylvestris*, respectively. The molecular weight (MW) standard was Fermentas100bp ladder in 1.5% agarose gels (**a**) and λ/HindIII in 6% polyacrylamide gel (**b**). Note the presence of unique bands in profile of *M.* ×*oxysepala* generated by OPH-15.

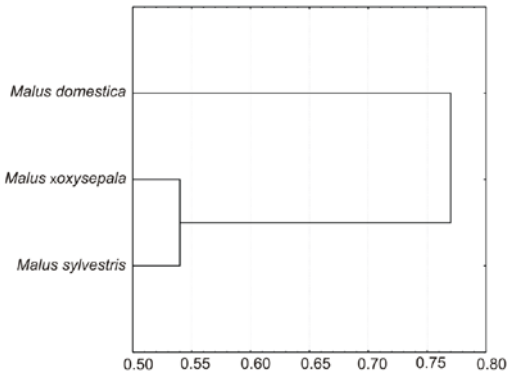

Fig. 9 Genetic relationships of the *Malus* species reflected in underweighted pair group method with arithmetic mean (UPGMA) dendrogram based on genetic similarity between *Malus sylvestris*, *M.* ×*oxysepala* and *M. domestica*.

Description

Trees of approximately 9 m in height. Leaf blades elliptic, less frequently obovate or orbicular, 3–11 cm in length and 2–7 cm in width, obtusely serrulate with fine crenation, clasping, at the base rounded or slightly cuneate with an acute tip, scarcely pubescent. Petiole of 1–5 cm in length. Flowers in pauciflorous umbels, white inside, pale pink outside, 3–4 cm in diameter, elliptic petals of 10–19 mm in length and 6–14 mm in width, peduncles and the calyx almost glabrous. Sepals acuminate, triangular 7–10 mm in length and 2–4 mm in width, 2.5–4 times longer than wide. The pentamerous pistil much longer than stamens. Fruits on short peduncles of 4–16 mm in length, orbicular or slightly orbicularly flattened, 3–5 cm in diameter, at the base and the tip emarginate, with a permanent cup, yellowish. Dark brown seeds, from 0 to 10 in one fruit, ovate of 5–7 mm in length and 4–5 mm in width and approx. 3 mm thick. Hilum located at the tip of seed, 1–2.2 mm in length and 0.5–0.8 mm in width. Seed surface macroscopically smooth and microscopically most frequently of four, five, six cells, closely arranged with marked margins and strongly bulged from 43 to 105 μm in length and from 22 to 32 μm in width. Phanerophyte, Mesophyte, Mesotrophic.

Holotype (one specimen) and isotype (one specimen) are collected in the herbarium of the Department of Botany in Poznań University of Life Sciences. Seeds (one bag) are collected in Carpological Collection in Department of Botany, Poznań University of Life Sciences.

Key

1.1 Mature leaves glabrous on the underside. Petals up to 20 mm in length. The ratio of sepal length to width up to 2.5. Fruits with a diameter of up to 35 mm. Seed sculpture with distinct, rimmed cells with straight anticlinal walls and bulged periclinal walls.....*Malus sylvestris*

1.2 Mature leaves puberulent. Petals up to 20 mm in length. The ratio of sepal length to width up to 3.5. Fruits with a diameter up to 50 mm. Seed sculpture with distinct, straight anticlinal walls and strongly bulged periclinal walls.....*Malus* ×*oxysepala*

1.3 Mature leaves on the underside tomentose. Petals up to 30 mm in length. The ratio of sepal length to width up to 2.5. Fruits with a diameter of up to 80 mm. Seed sculpture with distinct, rimmed cells with straight anticlinal walls and flat periclinal walls.....*Malus domestica*

Discussion

A novel species of a spontaneous hybrid *Malus* ×*oxysepala* was described from the area of Poland. Thus, there are three species spontaneously occurring in Poland, i.e. *M. domestica*, *M. sylvestris* and *M.* ×*oxysepala*. Despite the fact that morphological characters used to delimit species in series *Malus* often are continuous and overlapping [2], conducted morphological and genetic studies fully confirmed the hybrid character of the described species. Statistically significant differences between *M.* ×*oxysepala* and both parental species were found in somatic structures (leaves) and in reproductive structures (flowers, fruits, seeds). Some vegetative structures are highly susceptible to environmental influence – for example, the size of leaves and stems of many plants depends on whether shoots are exposed to sunlight or wind [21]. Therefore, results concerning leaves parameters should be treated with caution because observed differences might stem from environmental factors, which we did not control in our study. Our results suggest that the best qualitative traits are the seed surface sculpture observed under a scanning electron microscope (SEM) and leaves pubescence. One could not exclude that other diagnostic features can be

applied for the studied species. It would be worthwhile for future studies to analyze if diagnostics recently proposed by Ganeva and Uzunova [22] for section *Malus* and series *Malus*, namely cuticle ornamentation and wax deposition on leaves upper epidermis, could be applied for the studied hybrid species.

Molecular analysis confirmed previous phenotypic observations. Genetic profiles obtained for *M.* ×*oxysepala*, apart from unique bands, also show the presence of several products common for each of the assumed parents. However, on the basis of statistical analysis, we may observe a closer correlation of the hybrid with the wild species. This is not surprising in view of that indicate a considerable participation of *M. sylvestris* in the formation of new cultivars [17]. Generally, *M. sieversi* and *M. orientalis* are considered as the main ancestor for cultivated *M. domestica* [2,23,24]. On the other hand, studies based on the analysis of chloroplast DNA indicate much closer relations than previously believed between *M. sylvestris* and *M. domestica* in terms of the origin [25], which clearly indicates the possible spontaneous hybridization between them.

The presence of unique products in the profiles of the new taxon makes it possible to obtain specific SCAR (sequence-characterized amplified region) genetic markers, which, next to the morphological traits described above, would facilitate easy hybrid identification.

Acknowledgments

We are grateful to Prof. Jerzy Zieliński for Latin translation and two anonymous reviewers for critical comments on the manuscript. This research was supported by Ministry of Research and High Education Research Capacity Grants for Poznań Universiy of Life Sciences (No. 508.641.00 and 508.181.00).

Authors' contributions

The following declarations about authors' contributions to the research have been made: study conception: AC; collecting data: AC, RN; measurements and scanning: AC; morphological data analyses and interpretation: RN; molecular analyses and data interpretation: BG; writing the manuscript, table and figures arrangement: RN, BG, AC.

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