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GENE POOL VARIATION OF *Fragaria* × *ananassa* (Duch.) AND *Fragaria vesca* (L.)

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Abstract. Gene pool variation of twenty varieties and breeding clones of *Fragaria* × *ananassa*, nine varieties and breeding lines of *Fragaria vesca*, and one new interspecific hybrid designated *Fragaria* × *anavesca* was analysed with three DNA marker systems. ISSR reactions with four primers produced 45 polymorphic markers. Similarly, RAPD analyses with three primers produced 26 markers and SSR method with three primer pairs revealed 28 different alleles. The total number of 99 polymorphic markers allowed distinguishing clearly a group of *F.* × *ananassa* genotypes from that of *F. vesca* genotypes with *F.* × *anavesca* in between of these two. RAPD markers proved to be more informative than ISSRs as 3 of 26 were specific to *F.* × *ananassa* only and one exclusively to *F. vesca* and *F.* × *anavesca*. Thus, the presumed hybrid nature of *F.* × *anavesca* was effectively confirmed by RAPD markers. Especially important was the 1100bp long PCR product of the B104 primer present in all *F. vesca* genotypes as well as in *F.* × *anavesca* but absent in *F.* × *ananassa*. Presence of *F. vesca* DNA in the hybrid *F.* × *anavesca* was additionally corroborated by the 223bp product of the UDF017 primer pair and the 185bp-long band generated with the UDF006 primer pair.

Key words: *Fragaria,* strawberry, gene pool, interspecific hybrid

INTRODUCTION

The strawberry (*Fragaria* × *ananassa* Duch.) is an important fruit species cultivated widely across temperate and subtropical climate regions. Poland is one of the main producers of this fruit, which makes this species an important object of basic research and breeding. The strawberry arose from incidental hybridization of two American

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species: *F. virginiana* and *F. chiloensis* native to North and South America [Darrow 1966], respectively. First individuals of *F.* × *ananassa* appeared in Europe in the second half of the 18th century when plants of *F. chiloensis* were brought from Chile and planted near *F. virginiana*. Both ancestral components of the cultivated strawberry are natural autooctoploids. As a result of interspecific crossing between them, *F.* × *ananassa* has a very complex genetic structure of an autoallooctoploid (AAA'A'BBB'B'). Its genome consists of 56 chromosomes (2n = 8x = 56); [Darrow 1966, Bringhurst 1990]. Most probably, almost all contemporary varieties are direct descendants of one hybrid plant obtained in Europe over 250 years ago [Staudt 1961] with genetic variation coming from segregation and crossing-over events in a narrow gene pool with a relatively small component coming from mutations accumulated through the last two and a half centuries. Some cultivars however are reported to be obtained by backcrossing to one of the wild ancestors [Marta et al. 2004, Luby et al. 2008]. Studies of genetic diversity at the molecular level in the strawberry gene pool, as in the case of other crops, are of paramount importance to the breeders [Russell et al. 2004]. Crossing of genetically similar or closely related parents is not always an effective way of creating populations for subsequent selection and development of new cultivars. Unfortunately, in the case of many strawberry varieties and clones of interest, archival data on the ancestry are limited to only 2–3 generations backwards and knowledge of genetic differences between them is in fact very scarce or at least insufficient for development of efficient breeding programmes.

Fragaria vesca (L.) is the second species of economic importance in the genus *Fragaria*. It has much smaller fruits but it is widely appreciated for very attractive fruits taste and aroma, which are superior to those of *F.* × *ananassa*. *Fragaria vesca* has a diploid genome consisting of 14 chromosomes. The number of selected cultivars is relatively low when compared to *F.* × *ananassa* despite the fact that the species has been cultivated in Europe since Medieval times. A majority of the popular varieties were developed in Germany from the end of 19th to the middle of the 20th century. Although *F. vesca* has become a subject of intensive molecular research [Cekic et al. 2001, Sargent et al. 2004, Shulaev et al. 2011], the information on the variation within the species gene pool is practically non-existent.

In horticultural practice, the investigated species are multiplied in distinctively different ways. The varieties of *F. vesca* are inbred lines propagated by seeds while the varieties of *F.* × *ananassa* are vegetatively multiplied clones. This results in different approaches to the breeding of both species.

Wild forms of *F. vesca* as well as its cultivars are considered to be not only the source of genes controlling fruit taste and aroma but also other valuable traits like tolerance to abiotic stress and resistance to infectious agents. For example, in the Tatra Mountains (Southern Poland), wild *F. vesca* plants grow up to 1000 m above sea level and sustain harsh winter conditions (personal observation) likewise in the Bavarian Alps [Cornelius et al. 2013]. Distinct ecotypes of this species have been observed in the Novosibirsk region (Western Siberia, Russia) with a harsh continental climate by Baturin [2009]. Several attempts have been made to obtain hybrids between *F.* × *ananassa* and *F. vesca* with no evident success reported [Marta et al. 2004]. This was assigned to existence of prezygotic as well as postzygotic crossing barriers. Crosses made in an

opposite direction with *F. vesca* as a maternal component and *F. × ananassa* as a pollen parent appear to be more effective [Yanagi et al. 2010]. In the study, we included a new viable and fertile *F. × ananassa × F. vesca* hybrid obtained by us and designated *Fragaria × anavesca*. We hope that in the future it can be used as an effective intermediary in gene transfer procedures.

The purpose of the present study was a) to compare gene pool variation in the wild and cultivated strawberry with RAPD, ISSR and SSR markers, b) to identify species specific markers, and c) to provide molecular information on the newly obtained hybrid *Fragaria × anavesca*.

MATERIALS AND METHODS

Plant material. Leaves for DNA isolation were collected from twenty *F. × ananassa*, nine *F. vesca* genotypes, and one hybrid *F. × anavesca* (tab. 1). Among them, there were sixteen *F. × ananassa* commercial varieties of different age from very old ones to relatively new selections ('George Soltwedel', 'Aiko', 'Marmolada', 'Harunoka', 'Paros', 'Astra', 'Granda', 'Luna', 'Tango', 'Kaisers Samling', 'Toklat', 'Pai-Yu', 'Reikou', 'Eros', 'Heros', 'Ydun') and four breeding lines (2K27-1, Lwowskaja Rannaja S1-SR15, Pando WZ-4, Pau/27/27). The nine *F. vesca* genotypes comprised one developed from the wild line (collected in the Lublin region, Eastern Poland), one vegetatively propagated variety 'Minja', and seven clones that we obtained by the Single Seed Descent method [Goulden 1939] from commercial varieties; YeWo1, YeWo9, Reg2, BvS4, BvS5, Bvs-YGM, Rv4. All genotypes were randomly selected from a wide *in vitro* maintained collection. A new hybrid between *F. × ananassa* and *F. vesca* obtained by us and referred to as *Fragaria × anavesca* was included in our investigations.

DNA polymorphism analyses. DNA was isolated from lyophilized leaves using a double cycle of the Milligan [1992] method with RNAse treatment between the turns. RAPD analyses were performed in a 20μL volume containing 1 × PCR buffer (10 mM Tris-HCl pH 8.8 at 25°C, 50 mM KCl, 0.08% Nonidet P40), 2.5 mM $MgCl_2$, 200 μM dNTP, 0.6 mg of spermidine, 0.9 U Taq Rec (Fermentas) and 20 ng DNA, 200 nM of primer B104 (5'-ggg CAA TgA T-3') [Davis and Yu 1997], X01 (5'-Tgg CCA gTg A-3'), or X03 (5'-gTA gCT gAC g-3'). ISSR analyses were carried out as above with 0.75 mg of spermidine, 0.5 U Taq, 20 ng DNA and 400 nM of primer Is898 (5'-$[CA]_6$-RY-3'), IsJohn (5'-$[AG]_7$-YC-3'), IsTerry (5'-$[GTG]_4$-RC-3'), or Is901 (5'-$[GT]_6$-YR-3'). For SSR analyses, we used 1 × PCR buffer, 300 nM of each primer, 2.5 mM of $MgCl_2$, 200 μM of dNTP, 0.6 mg of spermidine, 0.6 U of Taq and 50 ng DNA in a 10 μL volume. We tested three SSR primers: UDF020 (F: 5'-ATgTCCATTTgCCgACATTT-3', R: 5'-gggTTTATTgggTTgggTTT-3'), UDF017 (F: 5'-ggACgTTCCACATCCgTAgA-3', R: 5'-CggTggAgATgTg-ATTTTATgA-3'), and UDF006 (F: 3'-CAggCAgT-TACTgAACTTACgg-5', R: 3'-AgAgTgCTCAgAgTCCATTgAT-5') [Cipriani and Testolin 2004]. The primers were selected based on their previous performance on a set of 125 clones of *F. × ananassa*.

In RAPD analyses, the following thermal profile was used: initial denaturation at 94°C for 2 min, 50 cycles at 92°C for 40 s, 38°C for 1 min 10 s, 72°C for 2 min with

Table 1. Origin and progeny of the *Fragaria* genotypes analysed in the study

No	Species	Name or designation	Country of origin	Descent and year of selection
1.	*F.* × *ananassa*	2K27-1	Poland	progeny of free pollinated cv. 'Oberschlesien'
2.	*F.* × *ananassa*	'George Soltwedel'	Germany	cv. 'Rotkäppchen' × 'Hansa' (1941)
3.	*F.* × *ananassa*	Lwowskaja Rannaja (S1-SR-15)	Poland	in vitro selected salt tolerant S_1 clone
4.	*F.* × *ananassa*	Pando (WZ-4)	Poland	progeny of free pollinated cv. 'Pandora'
5.	*F.* × *ananassa*	'Aiko'	USA (California)	CAL 46.5-1 × CAL 59.51-11 (1976)
6.	*F.* × *ananassa*	'Marmolada'	Italy	clone Nr 15 × cv. 'Gorella'
7.	*F.* ×*ananassa*	'Harunoka'	Japan	Kurume 103 × 'Dana' (1970)
8.	*F.* × *ananassa*	Pau/27/27	Poland	In vitro selected salt tolerant S_1 clone
9.	*F.* × *ananassa*	'Paros'	Italy	cv. 'Marmolada' × 'Irvine' (1998)
10.	*F.* × *ananassa*	'Astra'	Poland	cv. 'Dana' × 'Real' (1997)
11.	*F.* × *ananassa*	'Granda'	Poland	clone 83.5.8 × cv.'Marmolada' (1999)
12.	*F.* × *ananassa*	'Luna'	Poland	cv. 'Selva' × 'Real' (2001)
13.	*F.* × *ananassa*	'Tango'	UK	cv. 'Rapella' × 'Selva' (1994)
14.	*F.* × *ananassa*	'Kaisers Samling'	Germany	open pollinated 'Konig Albert von Sachen' (before 1912)
15.	*F. virginiana* / subsp. *glauca*	'Toklat'	USA (Alaska)	earlier classified as *F.* × *ananassa* (1977), in 2011 re-classified as *F. virginiana* subsp. *glauca*
16.	*F.* × *ananassa*	'Pai-Yu'	Taiwan	not known (before 1983)
17.	*F.* × *ananassa*	'Reikou'	Japan	not known (before 1994)
18.	*F.* × *ananassa*	'Eros'	UK	cv. 'Elsanta' × 'Allstar'(1994)
19.	*F.* × *ananassa*	'Heros'	Poland	cv. 'Gorella' × 'Dukat' (1996)
20.	*F.* × *ananassa*	'Ydun'	Denmark	not known (1948)
21.	*F.* × *anavesca*	*F.* × *anavesca*	Poland	*F.* × *ananassa* cv. 'Elsanta' × *F. vesca*
22.	*F. vesca*	(wild)	Poland	clone developed from wild growing plant collected in the Lublin area
23	*F. vesca*	YeWo1	Poland	progeny of cv. 'Yellow wonder'
24.	*F. vesca*	YeWo9	Poland	progeny of cv. 'Yellow wonder'
25.	*F. vesca*	Reg2	Poland	progeny of cv. 'Regina'
26.	*F. vesca*	'Minja'	Finland	*F.vesca* × *F.vesca* var. *semperflorens*
27.	*F. vesca*	BvS4	Poland	progeny of cv. 'Baron von Solemacher'
28.	*F. vesca*	BvS5	Poland	progeny of cv. 'Baron von Solemacher'
29.	*F. vesca*	Bvs-YGM	Poland	yellow-green leaf mutant found in progeny of cv. 'Baron von Solemacher'
30.	*F. vesca*	Rv4	Poland	progeny of cv. 'Reugen'

a final extension step of 72°C for 5 min. To generate ISSR markers, we used 4 min at 95°C, and 45 cycles at 94°C for 1 min, 46°C (Is901 and Is898) or 45°C (IsTerry and IsJohn) for 1 min, and 72°C for 1 min, with final extension of 5 min at 72°C. A standard SSR profile was used in the SSR analyses: initial denaturation at 95°C for 4 min, 7 cycles at 94°C for 45 s, 57°C (decreasing 1°C/cycle, TA + 7°C) for 45 s, 72°C for 45 s, 40 cycles at 94°C for 45 s, 50°C for 45 s, 72°C for 45 s with a final extension step of 72°C for 10 min.

The PCR products obtained in the SSR analyses were resolved on 5.5% polyacrylamide sequencing gels (350 × 450 mm) for 1 hour 10 min and silver stained [Chalhoub et al. 1997]. DNA markers pGEM® and SilverSTR® III Allelic Ladder Mix (Promega) were used to determine the size of the PCR products. The products of the RAPD and ISSR analyses were resolved on 1.5% agarose with 100 bp DNA Ruler™ (Fermentas) and stained with ethidium bromide. The results were documented with the BDA Digital (Biometra) system.

Statistical analyses. The bands were classified as polymorphic, species specific, and genotype specific within the panel of the genotypes studied. The polymorphic information content (PIC) for dominant markers was calculated to measure the informativeness with the formula:

$$PIC = 1 - [f^2 + (1 - f)^2],$$

where f is the frequency of the marker in the data set [Riek et al. 2001]. The polymorphic information content (PIC) for co-dominant markers was calculated according to the formula:

$$PIC = 1 - \sum_{i=1}^{k} P_i^2,$$

where k is the total number of alleles detected and P_i is the frequency of the i-th allele in the set of the *Fragaria* genotypes investigated [Weber 1990]. Principal components analysis (PCA) was performed with NT-SYS software [Rohlf 2001]. Genetic diversity (H) was computed for groups of *F.* × *ananassa* (FXA) and *F. vesca* (FVE) according to Lynch and Milligan [1994] with AFLP-SURV software [Vekemans et al. 2002].

RESULTS

In the ISSR analyses with 4 primers, 45 polymorphisms were obtained that did not differentiate clearly all the studied species (tab. 2). The PIC values of these dominant markers varied from 0.281 to 0.367. Similarly, 26 polymorphic bands were obtained with the 3 RAPD primers and the PIC varied from 0.259 to 0.309. However, the results were more informative as markers $B104_{880}$, $B104_{1050}$, and $X01_{910}$ were specific to *Fragaria* × *ananassa*, while marker $B104_{1100}$ was *F. vesca* specific (fig. 1). These markers provided evidence on the hybrid nature of *F.* × *anavesca*. Each of the co-dominant SSR markers targeted 2–3 loci and the average PIC values ranged from 0.447 (UDF020) to 0.747 (UDF006). The $UDF020_{160}$ allele was specific for *F.* × *ananassa*, while the $UDF017_{223}$ and $UDF006_{185}$ markers detected alleles invariable within *F. vesca* and not present in the *F.* × *ananassa* gene pool. The two latter markers provided additional evidence on the hybrid nature of *F.* × *anavesca*.

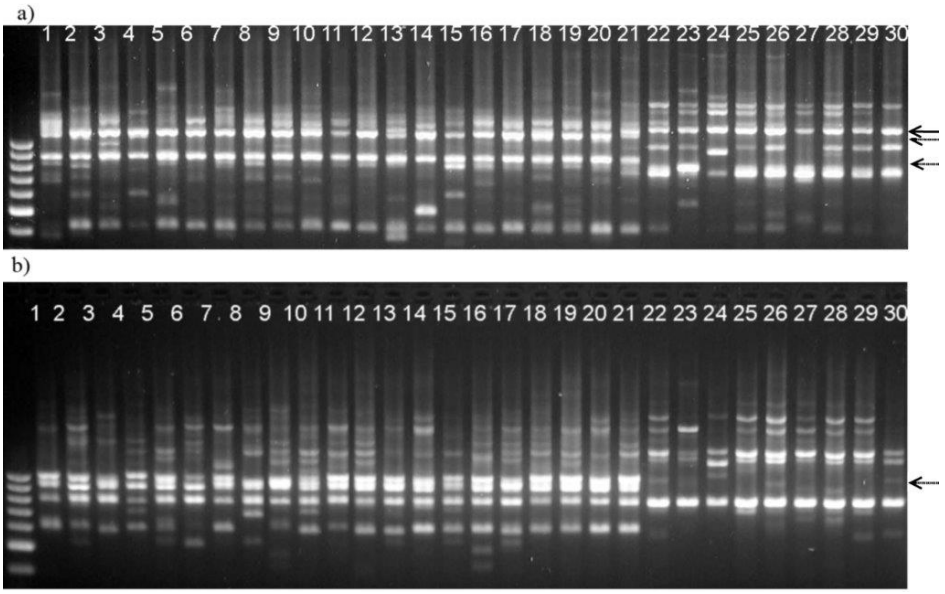

Fig. 1. Amplification patterns of RAPDs with primers B104 (a) and X01 (b). Lane numbers correspond to the material and method section: 1–20 represent the *F.* × *ananassa* gene pool, 21 – *F.* × *anavesca*, 22–30 are different genotypes of *F. vesca*. Solid and dotted darts indicate *F. vesca* specific and *F.* × *ananassa* specific markers, respectively

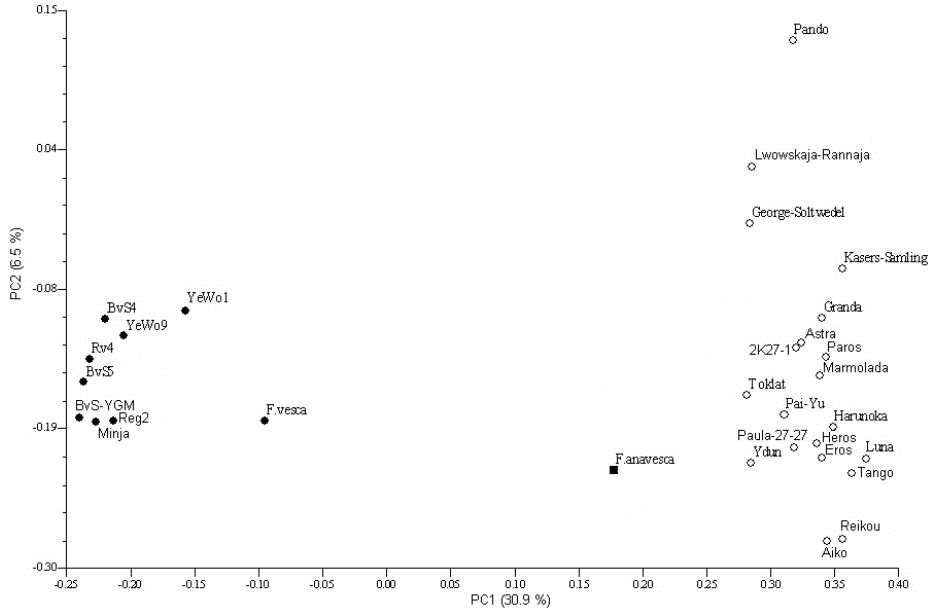

Fig. 2. Distribution of the 30 genotypes across the two main components of variation

Table 2. Characteristics of DNA polymorphism obtained with the markers tested

Marker system	Marker	Bands					PIC
		polymorphic	genotype specific	*F.* × *ananassa* specific	*F. vesca* specific	size min–max	
ISSR	Is901	13	3	0	0	430–1000	0.285
	IsTerry	13	2	0	0	380–930	0.281
	IsJohn	8	0	0	0	320–680	0.367
	Is898	11	0	0	0	420–1050	0.278
RAPD	B104	10	2	2	1	380–1100	0.302
	X01	8	2	1	0	480–1050	0.259
	X03	8	0	0	0	500–980	0.309
SSR	UDF020	9	0	1	0	129–177	0.464 0.278 0.598
	UDF017	8	2	0	1	155–237	0.731 0.278
	UDF006	11	3	0	1	168–204	0.788 0.706
Total		99	14	4	3		

Correlation coefficients computed between the 99 markers were used to extract the main components of variation. The two main principal components accounted for 30.9 and 6.5% of the overall DNA variation, respectively. Genotypes of both *F. vesca* and *F.* × *ananassa* formed distinct species–specific groups FVE and FXA, thus confirming linkage to two different gene pools (fig. 2). Group FVE was more compact then group FXA thus suggesting that the *F. vesca* genotypes represent lower genetic diversity when compared to the highly dispersed genotypes of the cultivated strawberry. *F.* × *anavesca* was located between these groups in proximity to FXA.

The overall genetic polymorphism was reduced within the FXA and FVE populations as 67.7 and 53.5% of the markers (with a frequency range of 5–95%) were polymorphic, respectively. The total Nei's gene diversity (Ht) was 0.365 and resulted equally from gene diversity within the population (Hw = 0.184) and between the populations (Hb = 0.181). Gene diversity within *F. vesca* was lower (0.153 ±0.017) than in the FXA group (0.216 ±0.017). The populations differed significantly; the observed Fst (0.491) was outside the p = 0.95 interval (from -0.03 to 0.12), as delimited by the permutation test with AFLP-SURV.

DISCUSSION

Markers obtained in result of application of ten primer sets allowed us to distinguish clearly all the thirty *Fragaria* genotypes in the study. Only two (clone 2K27-1 and cv. 'Astra') appeared to be very similar but still recognizable. *F. vesca* and *F.* × *ananassa*

formed two easily distinguishable groups with *F.* × *anavesca*, which was clearly separated from both of them. Three *F. vesca* clones developed from the 'Baron von Solemacher' variety were also widely separated on the graph (fig. 2). This was surprising because this species is generally supposed to be self-pollinated, which should result in high homozygosity and genetic uniformity of individuals within the variety. This assumption is strengthened by the observed morphological similarity of plants belonging to the given cultivar. A study conducted by Li et al. [2012] has shown that in natural conditions *F. vesca* ssp. *bracteata* produced 75% of seedlings by self-pollination. Our results suggest that the level of genetic variation within well-established commercial varieties of *F. vesca* is still relatively high and selection can give progress in breeding and bring improvement of at least some agronomically important traits. This diversity can be further broadened by crossing with another species, including octoploid *F.* × *ananassa*, which has been shown in our study as well as by Yanagi et al. [2010]. We also found a relatively long distance between the *F. vesca* clone collected from the wild in the Lublin region (eastern Poland) and all the other nine *F. vesca* clones in our study. This suggests existence of unexplored genetic resources that can be used in the future breeding programmes. Another interesting observation is the closeness of the cv. 'Toklat' to all the *F.* × *ananassa* varieties analysed. 'Toklat' [NCGR registration PI 616783 https://www.ars.usda.gov/SP2UserFiles/Place/53581500/catalogs/fracult.html] has been lately classified by the USDA National Clonal Germplasm Repository as *F. virginiana* subsp. *glauca*. Unfortunately, as we did not expect such an outcome, we did not include specimens of *F. virginiana* or *F. chiloensis* in our set of studied strawberry genotypes for comparison. As a result, our observations in this area are inconclusive. Finally, we found that despite its advanced age of nearly a quarter of the century, the RAPD marker system [Williams et al. 1990] is still a useful tool in gene pool studies. RAPD markers do not target any specific type of sequences but locate them according to their name randomly within the genome. RAPDs allowed us to identify the DNA of *F. vesca* origin in the genome of *F.* × *anavesca* and verified our earlier assumptions of its hybrid status.

CONCLUSIONS

1. The applied methods allowed us to distinguish clearly the investigated species and even very closely related clones of *F. vesca*.

2. Hybrid nature of the plant coming from the crossing between *F.* × *ananassa* and *F. vesca* was confirmed.

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ZMIENNOŚĆ W OBRĘBIE ZASOBÓW GENOWYCH *Fragaria* × *ananassa* (Duch.) I *Fragaria vesca* (L.)

Streszczenie. Analizowano zmienność w puli genów dwudziestu odmian i klonów hodowlanych *Fragaria* × *ananassa*, dziewięciu odmian i linii hodowlanych *Fragaria vesca* i nowego prawdopodobnego mieszańca międzygatunkowego nazwanego *Fragaria* × *anavesca* przy zastosowaniu trzech systemów markerów DNA. Cztery startery ISSR wygenerowały 45 markerów polimorficznych. Trzy startery RAPD dały 26 markerów, natomiast trzy startery SSR – 28 markerów polimorficznych. 99 uzyskanych markerów polimorficznych pozwoliło na jednoznaczne odróżnienie grupy genotypów należących do gatunku *F.* × *ananassa* od grupy genotypów należących do gatunku *F. vesca* z mieszańcem *F.* × *anavesca* sytuującym się pomiędzy nimi. Markery RAPD okazały się lepszym źródłem informacji niż ISSR, ponieważ 3 spośród 26 były specyficzne tylko dla *F.* × *ananassa*, a jeden wyłącznie dla *F. vesca* i *F.* × *anavesca*. W ten sposób, za pomocą markerów RAPD, potwierdzona została przypuszczalna wcześniej mieszańcowość *F.* × *anavesca*. Szczególnie istotny okazał się produkt reakcji PCR ze starterem B104 o długości 1100pz obecny we wszystkich genotypach *F. vesca* oraz *F.* × *anavesca*, a niewystępujący u *F.* × *ananassa*. Obecność DNA pochodzącego z *F. vesca* w mieszańcu *F.* × *anavesca* została dodatkowo potwierdzona poprzez produkt pary starterów UDF017 o długości 223pz i produkt pary starterów UDF006 o długości 185pz.

Słowa kluczowe: *Fragaria*, truskawka, pula genowa, mieszaniec międzygatunkowy

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