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Characterization of an allotriploid strawberry *Fragaria* × *bifera* Duchesne (Rosaceae) from Europe

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Abstract. Allopolyploidy has played an important role in the plant evolution. To assess its role in speciation, it is necessary to examine fertility and crossability of hybrids. A hybrid clone of the genus *Fragaria* with different and complex morphology compared to *F. vesca*, *F. viridis* and *F. moschata*, was detected in Germany (in Bayreuth, Bavaria). The genome size of these plants was measured using flow cytometry and their fertility was tested in experimental crossing. The parental origin of the hybrid was revealed using RAPD approach. From the mean intensity of fluorescence emitted by PI-stained nuclei for *F. moschata*, *F. vesca*, *F. viridis* and the hybrid, triploidy of the hybrid could be indicated. The hybrid shared an 1800bp and 880bp long species-specific RAPDs bands with *F. viridis* and *F. vesca*, respectively, indicating them as the parental species of the hybrid. The hybrid did not produce any fruit in selfing, open pollination and when crossed by pollen of *F. vesca* and *F. viridis*, all showing female sterility of the hybrid. The hybrid had 78% pollen sterility, however, pollinating *F. vesca* by pollen of the hybrid produced viable seed and F_1 plants, indicating its male fertility. This work shows allopolyploidy role in the evolution and speciation of *Fragaria*, and may suggest the study site as potential new centre of *Fragaria* speciation.

Key words: allopolyploidy, allotripolyploid strawberry, *Fragaria*, *Fragaria* × *bifera*, strawberry genome content

Izvleček. Karakterizacija alotriploidne jagode *Fragaria* × *bifera* Duchesne (Rosaceae) iz Evrope – Alopoliploidija ima pomembno vlogo v evoluciji rastlin. Kljub temu je za potrditev njene vloge pri speciaciji potrebno poznati plodnost in možnost križanja pri hibridih. V Nemčiji (Bayreuth, Bavarska) najden hibridni klon rodu *Fragaria* izkazuje drugačno in kompleksnejšo morfologijo kot vrste *F. vesca*, *F. viridis* in *F. moschata,* zato smo v analizi njegovega izvora uporabili molekulske pristope. Staršev ski izvor hibridov smo tako ugotavljali z metodo RAPD. Velikost genoma teh rastlin smo merili s pretočno citometrijo, plodnost hibridov pa smo testirali z eksperimentalnim križanjem. Povprečna intenziteta fluorescence *F. moschata*, *F. vesca*, *F. viridis* in hibrida, merjena s pretočno citometrijo, nakazuje triploidijo hibrida. Delitev 1800 in 880 bp dolgih vrstno specifičnih RAPD pasov z *F. viridis* in *F. vesca* pa nakazuje, da sta ti dve vrsti starševski hibridu. Hibrid ni proizvajal plodov pri samooploditvi, odprti oploditvi ali če je bil križan s pelodom *F. vesca* in *F. viridis*, kar nakazuje sterilnost ženskih hibridov. Hibridi so imeli 78 % sterilnost peloda, opraševanje *F. vesca* s pelodom hibrida pa je rezultiralo v viabilnih semenih in F_1 rastlinah, kar nakazuje na moško plodnost. Delo kaže na pomen alopoliploidije v evoluciji in speciaciji rodu *Fragaria* ter nakazuje možnost, da je lokacija študije nov center speciacije za rod *Fragaria.*

Ključne besede: alopoliploidija, alotripoliploidna jagoda, *Fragaria*, *Fragaria* × *bifera*, genom jagode

Introduction

Polyploidy has played an important role in the evolution of the flowering plants through adaptation and speciation (Grant 1981). Based on different estimations, 47% to 70% of flowering plants have polyploid origin (Grant 1981). Polyploidy can arise directly within single species (autopolyploidy) or through interspecific hybridisation involving duplication of chromosomes (allopolyploidy). Allopolyploidy is thought to be a predominant mode of speciation in flowering plants (Soltis & Soltis 1993, Rieseberg 1997). The formation and consequently fertilization of unreduced gametes during micro- and megasporogenesis is one of the main mechanisms creating polyploidy (Harlan & de Wet 1975, Bretagnolle & Thompson 1995).

The genus *Fragaria* comprises about 22 species (Staudt 2009), and includes different ploidy levels of diploids, tetraploids, hexaploid, octaploids (Staudt 1989) and decaploid along with several intermediate ploidy levels of interspecific hybrids (Bringhurst & Gill 1970, Staudt et al. 2003, Hummer et al. 2009, Lundberg 2011, Nosrati et al. 2011a, 2013). Three *Fragaria* species, i.e. the diploids *F. vesca* L., *F. viridis* Weston and the hexaploid *F. moschata* Weston, are distributed in Europe (Staudt 1962, Tutin et al. 1968) along with several interspecific hybrids originating between them, including tetraploids *F.* × *intermedia* (Bach) Beck (between *F. moschata* and *F. vesca*) and *F.* × *neglecta* Lindem (between *F. moschata* and *F. viridis*) and diploid *F.* × *hagenbachiana* K. H. Lang ex W. D. J. Koch (between *F. viridis* and *F. vesca*), triploid *F.* × *bifera* Duchesne between *F. vesca* and *F. viridis* (Staudt et al. 2003), pentaploid (Nosrati et al. 2011a) and heptaploid (Nosrati et al. 2013) hybrids both between *F. vesca* and *F. moschata*. Moreover, interspecific tetra-, penta- and octoploid hybrids have also been artificially produced between European species (Lippert 1985).

Morphological recognition among species in the genus *Fragaria* is very difficult even at interploidy levels because of high morphological variations and similarities (Ichijima 1930). This difficulty is even higher in interspecific hybrids, especially hybrids between *F. vesca* and *F. viridis* due to close genetic relationship and higher morphological similarities (Potter et al. 2000).

To assess the likelihood that a new interspecific polyploid hybrid will be successfully established, it is necessary to have information on the viability and fertility of the hybrids (Ramsey & Schemske 1998). In almost all cases, the fertility and crossability of the interspecific hybrids reported so frequently in literature and papers have not been documented.

This work is aimed at documenting a putative interspecific triploid hybrid *Fragaria* from Europe on the basis of fertility, crossability, genome size, and DNA fingerprinting.

Materials and methods

Plant material

A clone of *Fragaria* with morphology different from the three European species *F. vesca*, *F. viridis* and *F. moschata* was detected in Germany, Bavaria (around Bayreuth, between Destuben and Rodensdorf; latitude: 49° 54' 10" N; longitude: 11° 34' 16" E), where all three species grow together. The preliminary investigations introduced it as a potential putative interspecific hybrid plant. This putative hybrid *Fragaria* had complex morphology so that its definite recognition was not possible on the basis of morphological characters. For example, runners in *F. viridis* are always monopodial, short and filiform, while in *F. vesca* they are sympodial and long (Tutin et al. 1968). The putative allotriploid hybrid *Fragaria* had runners of both types. The putative hybrid along with some samples of all the species growing in the site of the hybrid were transferred to the glasshouse at the University of Aberdeen, UK, for further analyses of total genome size to reveal the ploidy level using flow cytometry, fertility levels for understanding the extent of reproductive isolation between putative hybrid and its parental species using crossing experiments, and recognition of parental origin using RAPDs.

Genomic DNA measurement

The total amount of nuclear DNA of the putative hybrid along with several samples of *F. vesca, F. viridis* and *F. moschata* as putative parental species was measured using flow cytometry. We used the well-known cultivated octoploid strawberry *F.* × *ananassa* cv. Vivorosa as internal DNA reference standard. The chicken erythrocytes were used to test the linearity of the system. Approximately 100mg young leaf tissue was chopped with sharp scalpel in 1ml of ice-cold nuclear isolation buffer, LB01 (Dolezel et al. 1992). This buffer consisted of 15 mM Tris, 2 mM Na_2EDTA, 80 mM KCl, 20 mM NaCl, 0.5 mM spermine, 15 mM b-mercaptoethanol, 1 ml/l Triton X-100 with the modification of adding PVP-40 at the proportion of 10 g/l (Yokoya et al. 2000) as (1%) PVP-40 in the chopping solution which has been shown to increase the number of intact nuclei isolated in flow cytometric studies of Rosoideae (Dickson et al. 1992). Final pH was adjusted to 7.5. A volume of 0.5 ml lysate was recovered, after filtering through nylon gauze (pore size 50 µm). Ribonuclease A (2.9 µl of a 34 mg/l solution) and propidium iodide (PI) (10 µl of a 20 mg/l solution) were added and the lysate incubated in the dark for 1–1.5 h on ice. The samples were filtered through nylon gauze (pore size 50 or 20 µm) just before measuring, and fluorescence intensity was measured with a Becton Dickinson FACSCalibur Benchtop Cytometry Analyser. Domestic chicken lymphocytes were used as an internal DNA reference standard of known genome size following Galbraith et al. (1983). The PI-stained nuclei were excited by a 488 nm laser, and mean fluorescence intensity (MFI) of fluorescence emitted by nuclei was recorded. The total amount of nuclear DNA was assessed relative to that of cultivated *F.* × *ananassa* cv. Vivorosa (2n = 8x = 56) setting the MFI peak at 800.

Fertility test and crossing experiments

The pollen sterility of putative hybrid and parental species were tested on the basis of percentage of unstained pollen with 0.05% cotton blue in lactophenol by examining a minimum of 520 pollen grains. The fertility of putative hybrid along with putative parental species was measured on the basis of the fruit set under open pollination in the glasshouse over a period of 2.5 years. In addition, male and female fertility of the putative hybrid were investigated in artificial reciprocal experimental crosses between the putative hybrid and the three *Fragaria* species growing in the hybrid site. For hand-crossing experiments, floral buds on seed parents were emasculated approximately 3—4 days before anthesis by removing the indehiscent anthers with a sharp scalpel. These anthers-emasculated flowers were inspected using a *x*10 hand lens to ascertain that they were un-dehisced. The anther-emasculated flowers were gently washed with a little water and, after air-dried, pollinated by directly rubbing the anthers from the pollen parent onto the exposed stigmas. The hand-pollinated flowers were immediately covered by the double-layer of fibre fleece in order to prevent uncontrolled open-pollination and desiccation. Hand pollination was repeated a second time after 1—3 days to ensure the presence of viable pollen on stigma at the time of stigmatic receptivity.

Artificial hand-crossing was also carried out between putative parental species in order to measure the fertility levels and genome size among originated F_1 hybrids. Consequently, the fertility and genome size of the artificial hybrids were compared with those of the natural putative hybrid under study.

RAPDs analysis for parentage identification

First, species-specific RAPD markers were established for the three *Fragaria* species growing in the site of the putative hybrid using some 10 arbitrary RAPD primers. Consequently, the occurrence of the species-specific RAPD markers was investigated in the putative hybrid (Tab. 1). The RAPDs patterns in each case were repeated to ensure the reproducibility of the PCR profiles. The documentation of the hybrid and its parental origin was based on the presence of species-specific RAPDs markers of two different species in the putative hybrid.

Results

Genomic DNA content

The genomic DNA content of the putative hybrid and the three species of *F. vesca*, *F. viridis* and *F. moschata* growing in the site of the hybrid, along with the octoploid *F.* × *ananassa* cv. Vivorosa used as a control are shown in Fig. 1 and Tab. 1. In the flow cytometric assessment, when the MFI for the octoploid *F.* × *ananassa* cv. Vivorosa set at 800, this value, on average, for hexaploid *F. moschata*, diploids *F. vesca* and *F. viridis* was 613.64, 228.5 and 232.4, respectively, while the MFI value for the putative hybrid was found to be 309.9.

Table 1. Flow-cytometric mean fluorescence intensity (MFI) of genomic DNA content indicating the ploidy level in putative allotriploid hybrid *Fragaria* and samples of the three putative parental species from the study site.
Tabela 1. Povprečna intenziteta fluorescence (MFI) pri pretočni citometriji vsebine genomske DNA, ki nakazuje na stopnjo plidnosti v verjetnem alotriploidnem hibridu rodu *Fragaria* in za vzorce treh domnevnih starševskih vrst z istega območja raziskave ter kultiviranega oktoploida *F.* × *ananassa*.

Taxon	Mean MFI (CI*)
F. moschata Duchesne	613.64 (4.71)
F. viridis Watson	228.50 (8.08)
F. vesca L.	232.40 (8.00)
putative hybrid	309.90 (7.45)
F. × ananassa cv. Vivorosa	800

*CI (95%): confidence interval

RAPDs patterns

Out of the ten RAPD primers applied for establishing species-specific markers for the three species growing in the hybrid site, only four primers produced unique bands for the species. The primer D produced one unique band specific to *F. viridis*, and one unique band specific to *F. vesca*. In addition, the primer A produced one unique band specific to *F. vesca*. Two species-specific bands were detected in the RAPD patterns of the putative triploid hybrid, of which one band specific to *F. viridis* with band size of 1800bp and one band specific to *F. vesca* with band size 880 bp (Fig. 2).

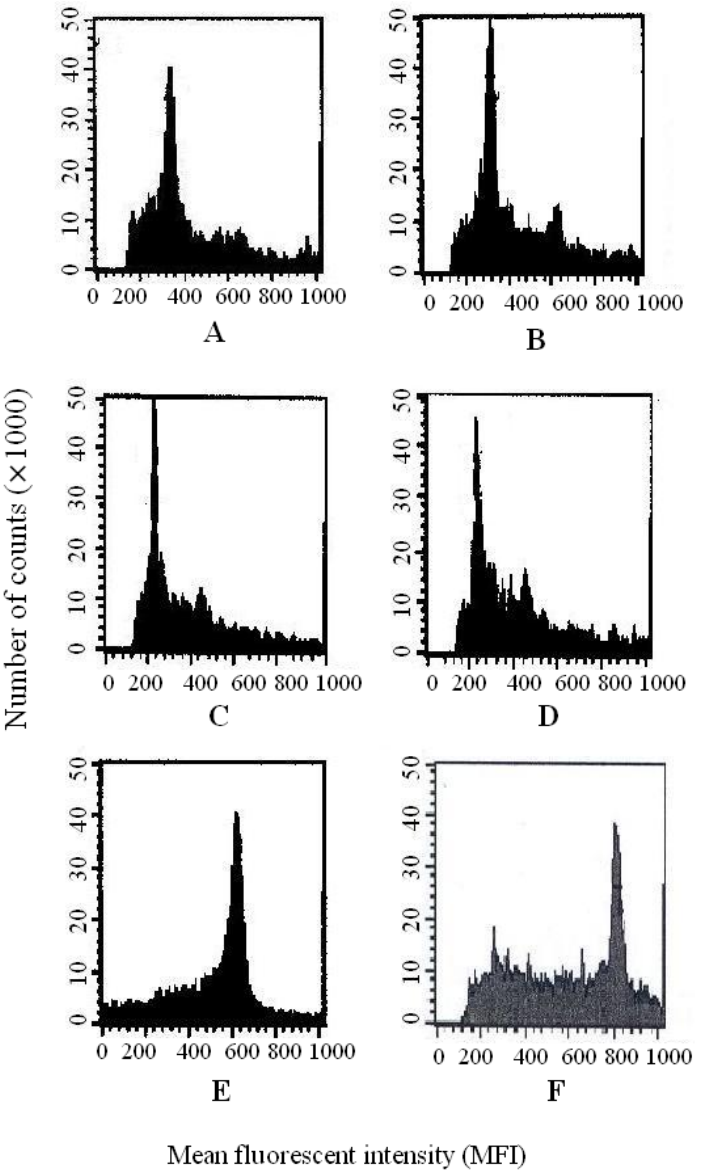

Figure 1. Samples of flow cytometry histograms showing genomic DNA content in the putative hybrid *Fragaria* (A, B) having originated from between *F. vesca* (C) and *F. viridis* (D) in Bayreuth, Bavaria, Germany along with hexaploid *F. moschata* (E) and the cultivated octoploid *F. × ananassa*.

Slika 1. Primeri historamov pretočne citometrije, ki kažejo vsebino genomske DNA v domnevnem hibridu *Fragaria* (A, B), ki izvira iz križanja med *F. vesca* (C) in *F. viridis* (D) v kraju Bayreuth, Bavarska, Nemčija, skupaj s heksaploidnim *F. moschata* (E) in kultiviranim oktoploidom *F. × ananassa* (F).

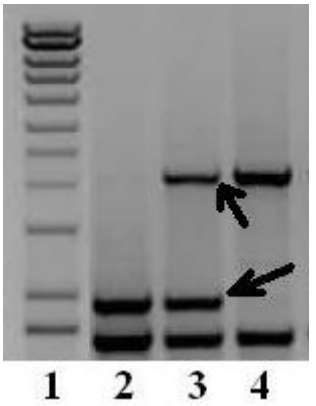

Figure 2. RAPDs pattern of the allotriploid hybrid (lane 3) and its putative parental species, i.e. *F. vesca* (lane 2) and *F. viridis* (lane 4), using primer D (sequence: 5'GTCCTTAGCG3'). The hybrid shared a band of 880 bp size (lower arrow) with *F. vesca* and a band of 1800 bp size (upper arrow) with *F. viridis* (The lane codes: 1 = standard size markers, 2 = *F. vesca*, 3 = the allotriploid hybrid, 4 = *F. viridis*).

Slika 2. Vzorec RAPD alotriploidnega hibrida (tretja kolona) in njegovih domnevnih starševskih vrst, t. j. *F. vesca* (stolpec 2) in *F. viridis* (stolpec 4), z uporabo primerja D (sekvenca: 5'GTCCTTAGCG3'). Hibrid si je delil pas 880 baznih parov (spodnja puščica) s *F. vesca* in pas 1800 baznih parov (zgornja puščica) s *F. viridis*. Stolpec 1 so standarizirane velikosti markerjev.

Fertility and crossing experiments

The putative hybrid had at least 78% pollen sterility, while the values for putative parental species of *F. vesca* and *F. viridis* were 6.8% and 23%, respectively. The pollen sterility for hybrids artificially obtained from crossing *F. vesca* with pollen of *F. viridis* was on average 34% (Tab. 2). These artificial interspecific hybrids were found to be diploid using flow cytometry.

Table 2. Pollen sterility in putative allotriploid hybrid and putative parental species.
Tabela 2. Sterilnost peloda pri domnevnem alotriploidnem hibridu in pri domnevnih starševskih vrstah.

Species and hybrids	No. of pollen examined	% pollen sterility	C.I. (95%)
F. vesca	703	6.8	1.9
F. viridis	521	23	3.6
Artificial hybrids*	843	34	3.2
Putative allotriploid	688	78	3.0

* Artificial hybrids were made between *F. vesca* and *F. viridis*.

In crossing experiments, crossing the putative hybrid by pollen of both *F. vesca* and *F. viridis* did not result in any fruit (cross types 1 and 2 in Tab. 3). In addition, hand-selfing of 24 flowers of the allotriploid hybrid also did not set any fruit, and similarly, 172 flowers of the hybrid plant did not produce any berry in open pollination at the glasshouse in a period of over 2.5 years (Tab. 3). However, crossing 7 flowers of *F. vesca* by pollen of the hybrid produced 2 berries (cross type 3 in Tab. 3). These two berries producing the total number of 21 seeds (achenes) had 15% and 17% levels of seed set. Only 9 out of 21 seeds germinated and produced seedlings, of which 5 matured in the glasshouse.

Table 3. Artificial crosses made between the putative hybrids and the putative parental species from the site of the hybrid, as well as among putative parents.
Tabela 3. Rezultati umetnih križanj med domnevnimi hibridi in domnevnimi starševskimi vrstamu z najdišča hibrida, kot tudi med domnevnimi starševskimi vrstami.

Cross type	Crosses (seed parent × pollen parent)	No. of crosses	No. of berries	% fruit set	% seed set	No. of achenes		No. of mature plants
						produced	germinated	
1	putative hybrid × *F. vesca*	5	0	-	-			
2	putative hybrid × *F. viridis*	1	0	-	-			
3	*F. vesca* × Putative hybrid	7	2	29	22 & 23	21	9	5
4	selfing of Putative hybrid	24	0	-	-			
5	putative hybrid in Open pollination	172	0	-	-			
6	*F. vesca* × *F. viridis*	12	9	75				
7	*F. viridis* × *F. vesca*	4	0	-	-			

Discussion

In this study, the ploidy level, fertility and parental origin of an allotriploid hybrid strawberry between diploids *F. vesca* and *F. viridis* was documented in Bayreuth, Bavaria (Germany) based on flow cytometric measurement of the genome size, the artificial crossing experiments, monitoring the outcome of open pollination during 2.5 years at the glasshouse, and detection of parental origin using RAPD markers. The triploidy of the hybrid indicates that an unreduced gamete was most likely involved in the formation of this allotriploid hybrid through sexual reproduction.

The artificial reciprocal crosses made in this work between putative parental species, i.e. *F. vesca* and *F. viridis*, were almost only successful in one direction, when *F. vesca* was used as seed parent. This is consistent with our previous work based on tremendous mutual crosses between these diploids, which showed that interspecific crosses between *F. vesca* and *F. viridis* were successful almost always when *F. vesca* was used as seed parent (Nosrati et al. 2011b). Therefore, it can be concluded that in the formation of allotriploid hybrid characterized in the current study between *F. vesca* and *F. viridis*, the latter species played as pollen donor parent.

The interspecific allotriploid hybrid reported in the current work was female sterile, as crossing it by viable pollen of *F. vesca* and *F. viridis* was unsuccessful. Moreover, the hybrid did not set any fruit in many hand-selfing crosses neither under open pollination over 2.5 years at the glasshouse. However, backcrossing *F. vesca* by pollen of the allotriploid hybrid produced fruit with viable seeds and offspring.

As we previously showed that hybrids originating from artificial hand-crossing experiments between *F. vesca* and *F. viridis* were almost always homoploid hybrids, i.e. diploids (Nosrati et al. 2011b), it can be concluded that naturally occurring allotriploid hybrid between these two species is, in fact, very rare.

The putative allotriploid hybrid reported in the current work had very high pollen sterility (78%), while this value for the artificial diploid interspecific hybrid obtained in the current work between *F. vesca* and *F. viridis* was low (34%). However, using the same technique and the other accessions of *F. vesca* and *F. viridis*, the level of pollen sterility among F_1 interspecific hybrids originated between these species was on average 65% with standard deviation of 1.8 (Nosrati et al. 2011b). This indicates that allopolyploids and homoploid hybrids may have the same level of male sterility.

The two natural hybrids, i.e. diploid *F.* × *hagenbachiana* K. H. Lang ex W. D. J. Koch and triploid *F.* × *bifera* Duchesne (Staudt et al. 2003), have already been reported between *F. vesca* and *F. viridis*, however, the levels of sterility in the hybrids and the occurrence and levels of reproductive isolation between hybrids and their parental species were not investigated. Therefore, the possibility that the previously reported hybridization between *F. vesca* and *F. viridis* will result in speciation, cannot be interpreted.

In the evolution and speciation of *Fragaria*, allopolyploidy has already played a vital role in different events, such as the formation of hexaploid *F. moschata* via two allopolyploid occasions among three diploids *F. vesca*, *F. viridis*, and *F. iinumae* Makino, and in the formation of octoploid *Fragaria* via allopolyploidy between *F. moschata* and *F. iinumae* (Lundberg 2011). Similarly, we have previously reported a natural allopentaploid and alloheptaploid hybrids both between *F. moschata* and *F. vesca* from this site (Nosrati et al. 2011a, 2013).

Hybrid formation involving contribution of functioning unreduced gametes have been frequently reported in the genus *Fragaria* from both wild (Bringhurst & Senanayake 1966, Bringhurst & Gill 1970, Staudt et al. 2003) and artificial crossing experiments (Fedorova 1934, Scott 1951, Ellis 1962, Staudt 1962). They indicated the vital role of the functioning unreduced gametes in the evolution of polyploidy in *Fragaria*. The frequency of functioning unreduced pollen in flowering plants was estimated to be 0.05% (Ramsey & Schemske 1998). A higher levels of polyploid F_1 progeny originating from artificial intraploid crosses have been reported in the flowering plants, e.g. onion (Jones & Clarke 1942), orchids (Storey 1956) and cassava (Hahn et al.1990). These may suggest that unreduced gametes may play an important role in interspecific hybridization, and consequently allopolyploidy evolution.

The results of the current study confirming our previous reports (Nosrati et al. 2011a, 2013) show that allopolyploidy still plays an important role in the evolution and speciation of the genus *Fragaria*, and that the study site could act as a new centre for speciation of *Fragaria*.

Povzetek

Ker alopoliploidija igra pomembno vlogo v evoluciji in speciaciji rastlin, je potrebno raziskati tudi plodnost in možnost križanja hibridov. Klon rodu *Fragaria* z drugačno morfologijo je bil najden v Nemčiji (Bayreuth, Bavarska), na rastišču, kjer sicer uspevajo tri evropske jagode, t.j. *F. vesca*, *F. viridis* in *F. moschata.* Analiza RAPD je pokazala, da je nova jagoda hibrid in da izvira kot vmesna oblika med *F. vesca* in *F. viridis*, saj z obema evropskima diploidoma deli vrstno specifične lokuse. Meritve velikosti genoma hibrida z uporabo pretočne citometrije je pokazala triploidno garnituro kromosomov hibrida. Kot kaže ima hibrid sterilne ženske rastline, saj ni sposoben proizvajanja plodov pri prostem opraševanju, umetno samooploditvijo ali pri križanju s pelodom predvidenih starševskih vrst. Moške rastline so plodne, saj je oprašitev *F. vesca* s pelodom hibrida rezultirala v viabilnih semenih in F_1 rastlinah. Študija potrjuje pomen alopoliploidije v evoluciji in speciaciji rodu *Fragaria*, rastišče hibrida pa bi bilo lahko nov center speciacije rodu *Fragaria*.

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