UDC (UDK): 634.75

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PHYLOGENETIC RELATIONSIP AMONG SPECIES OF FRAGARIA BASED ON RAPDS

SUMMARY

The genus *Fragaria* L. (strawberry, Rosaceae) comprises of 22 species of different ploidy. The ploidy evolution in *Fragaria* is not clear. The phylogeny of *Fragaria* was investigated using RAPDs. The ploidy level was estimated from DNA content using flow cytometry. Genetic distance was calculated using Squared-Euclidean Distance, and cluster analysis constructed using Between-Groups Linkage. Four out of ten examined primers produced 68 polymorphic and reproducible bands. The lowest genetic distance (1.7) was found between *F. vesca* ssp. *vesca* and *F. vesca* ssp. *vesca* f. *monophylla*, while distances of *F. vesca* ssp. *vesca* from *F. vesca* ssp. *californica* and *F. vesca* ssp. *americana* were 10.3 and 12.3, respectively. This distance between *F. vesca* ssp. *vesca* and the diploids *F. viridis, F. nubicola, F. daltoniana, F. nipponica* and *F. nilgerensis* ranged from 14.3 to 24.3. The greatest distances between *F. vesca* ssp. *vesca* and the octoploids were 39 - 44.3. Cluster analysis grouped the species in two groups: all octoploids in one cluster, and diploids alongside tetraploid and hexaploid in another cluster. The data showed a close relationship between *F. orientalis* and *F. moschata* and also among octoploids *F. virginiana*, *F. chiloensis* and *F. x ananassa,* and proposed *F. x ananassa* as hybrid between *F. virginiana*, *F. chiloensis*. This work supports the current taxonomy of octoploid at subspecies level, and disagrees with morphometric and ITS/trn data, and suggests that polyploidy in *Fragaria* has occurred twice: once resulting in tetraploid and hexaploid in Eurasia and in another event produced the octoploids in America.

Keywords: *Fragaria*, strawberry, phylogenetic relationship, polyploid evolution

INTRODUCTION

The genus *Fragaria* L. (strawberry, Rosaceae) is a perennial herb comprising of about 22 species, some of which include several subspecies. The base chromosome number in *Fragaria* is 7 and ploidy levels include diploid, tetraploid, hexaploid and octoploid (Staudt, 1989; Staudt, 2008). The genus is of mostly Holarctic distribution occurring all over the North Temperate regions with diploid *F. vesca* has a world-wide distribution throughout northern Eurasia and both North and South America (Staudt, 1999).

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Understating the ploidy evolution in the genus *Fragaria* is difficult due to lack of intermediate ploidy levels. In Europe diploids *F. vesca* and *F. viridis* are sympatrically distributed alongside the hexaploid species *F. moschata*, where the intermediate tetraploid absence, while tetraploid *F. orientalis* occurs in Asia (Staudt 1989, 1962). Similarly, several diploid species including *F. vesca* ssp. *americana* and *F. vesca* ssp. *californica* alongside the three octoploids *F. virginiana, F. chiloensis* (and their natural interspecific octoploids hybrid *F. x ananassa*) are distributed in New World, where the intermediate ploidy levels (i.e. tetraploid and hexaploid) do not occur. The previous study on phylogenetic relationships in *Fragaria* was based on restriction fragment variations of chloroplast DNA (cpDNA) using nine *Fragaria* species (Harrison *et al*., 1997a). This study revealed a little information on the phylogenetic of *Fragaria*. However, it showed that the Asian tetraploid *F. orientalis* and the European hexaploid *F. moschata* are sisters, and so are the American octoploids *F. virginiana* and *F. chiloensis*. Moreover, based on the evidence obtained from natural hybridization and experimental crosses as well as similarities inferred from chromosomes structures, phylogentic relationship were suggested among some *Fragaria*. For instance, a close relationship between *F. vesca* and *F. chiloensis* was inferred on the basis of their interspecific natural hybrid located in California (Bringhurst and Khan, 1963; Bringhurst. 1990). Studying relationship among subspecies of two American octoploids based on RAPD data indicated a closer relationship of *F. virginiana* subsp. *platypetala* to *F. chiloensis* than to other subspecies of *F. virginiana* (Harrison *et al*., 1997b). Later, Potter *et al*. (2000) using chloroplast DNA/*trn* and nuclear ITS data confirmed Harrison *et al*. (1997b) conclusion that *F. virginiana* ssp. *platypetala* is more closely related to *F. chiloensis*.

In order to insight to the phylogeny of *Fragaria,* this work attempted to investigate the relationship among species of *Fragaria* using RAPD markers.

MATERIAL AND METHODS

A wide range of *Fragaria* species comprising of all its ploidy levels e.g. diploids, tetraploid, hexaploid and octoploids were investigated. In addition, several subspecies, forma and/or genotypes from diploid *F. vesca* and octoploid *F. chiloensis* and *F. virginiana* were included in this study in order to assess the RAPDs ability in recognizing taxa at lower taxonomic levels (Table 1).

The ploidy level of the plants was estimated on the basis of nuclear DNA content using flow cytometry. The cultivated octoploid strawberry *F.* x *ananassa* cul. *Vivorosa* was used as internal DNA reference standard, and the chicken erythrocytes implemented for testing the linearity of the system. Approximately 100mg young leaf tissue was chopped with sharp scalpel in 1ml of ice-cold nuclear isolation buffer of LB01, which consists of 15mM Tris, 2mM Na_2EDTA, 80mM KCl, 20mM NaCl, 0.5mM spermine, 15mM b-mercaptoethanol, 1ml/l Triton X-100 (Dolezel *et al*., 1992) with minor change of adding PVP-40 at the proportion of 10g/l in order to increase the number of intact nuclei isolated

(following Dickson *et al*., 1992; Yokoya *et al*., 2000), by adjusting the final pH at 7.5. A volume of 0.5ml lysate was recovered, after filtering through nylon gauze (pore size 50µm). Ribonuclease A (2.9µl of a 34mg/l solution) and propidium iodide (PI) (10µl 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 20µm), and then were assessed for fluorescence intensity 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 488nm 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.* x *ananassa* cul. *Vivorosa* (2n = 8x = 56) setting the MFI peak at 800 since flow cytometry provides a relative value compared to a reference of known ploidy.

Table 1. Source and accessions number of plant material used in this study.

Plant No.	Species	Ploidy level	Source + determination/accession number
1	*F. virginiana* ssp. *platypetala* (Rydb.) Staudt	8x	NCGR/426.000 PI 551786
2	*F. virginiana* ssp. *virginiana*		NCGR/1250.001 PI 616608
3	*F. virginiana*		Agroforestry Research Trust, Dartington, Devon, UK
4	*F. chiloensis* ssp. *chiloensis*		NCGR/1107.002 PI 602569
5	*F. chiloensis*		W E Th Ingwersen LTD, East Gravetye, England
6	*F.* x *ananassa* cul. Vivorosa		BQ, Aberdeen, UK
7	*F.* x *ananassa* cul. Elsanta		BQ, Aberdeen, Uk
8	*F. vesca* ssp. *americana* (Porter) Staudt	2x	NCGR/554.001 PI 551881
9	*F. vesca* ssp. *Californica* (Cham. et Schlecht.)		NCGR/371.001 PI 551749
10	*F. vesca* ssp. *monophylla*		NCGR/612.000 PI 551909
11	*F. vesca* ssp. *vesca*		NCGR/Kew botanic garden, England
12	*F. vesca* ssp. *vesca*		NCGR/197.000 PI 551572
13	*F. vesca* ssp. *vesca*		NCGR/478.000 PI 551826
14	*F. vesca* ssp. *vesca*		NCGR/198.000 PI 551573
15	*F. vesca* ssp. *vesca*		University of Joensuu, Finland
16	*F. vesca* ssp. *vesca*		Poyntzfieldherbs Nursery, Inverness, UK
17	*F. viridis* Weston		NCGR/1256.001 PI 616609
18	*F. iinumae*	??	NCGR/538.001 PI 551866
19	*F. nipponica* Makino	2x	NCGR/540.001 PI 551868
20	*F. nilgerrensis* Schldl. ex J. Gay		NCGR/1223.001 PI 616602
21	*F. daltoniana* J. Gay,		W E Th Ingwersen LTD, East Gravetye, England
22	*F. nubicola* (Hook. f.) Lindl.		Poyntzfieldherbs Nursery, Inverness, UK
23	*F. orientalis*	4x	NCGR/538.001 PI 551866
24	*F. moschata*	6x	NCGR/541.000 PI 601869

Genomic DNA was extracted from approximately 100mg fresh leaf material of plants using in liquid N_2 by DNeasy Plant Mini Kit (Qiagen). Out of ten PCR primers (Invitrogen) examined, four primers were selected due to producing a higher number of polymorphic clear and reproducible bands (primers sequence (5' to 3') D: GTCCTTAGCG, F: CTACTACCGC, G: GAGTCACTCG, H: GTCCTCAGTG). PCR reactions were carried out in 25*µl* volumes containing 1x (2.5µl) PCR buffer, 1U (0.2µl) *Taq* DNA polymerase, 200µM (0.5µl) dNTPs, 1µM (0.5µl) primer, 25ng (5µl) DNA target, and 2.5mM (1.25µl) $MgCl_2$ with adding 15µl sterilised water. PCR amplifications were conducted in a PCR Express thermal cycler (Hybrid UK) with the following temperature profile:

94°C for 1 min, 45°C for 1 min, 72°C for 2 min for 36 cycles, with a final extension at 74°C for 7min. PCR amplification products were separated on 1% (w/v) agarose gel, and stained by ethidium bromide stained. The banding patterns of the plants were scored as 1 and 0 for the presence and absence of bands, respectively.

Genetic distance between the plants was calculated between pairs of plants using Squared Euclidean Distance based on RAPD markers. Since genetic distance between six accessions of *F. vesca* ssp. *vesca* used in this study was variable, the genetic distances obtained within this taxon were averaged between the 15 pairs of six accessions to obtain one value. The genetic distance between *F. vesca* ssp. *vesca* and all other taxa were obtained by averaging the distances between each of six accessions of *F. vesca* ssp. *vesca* and the taxon in question. A dendrogram was constructed using the cluster method of Between-Groups Linkage (SPSS version 13.1) for polymorphic RAPD markers to show the relationship between taxa.

RESULTS AND DISCUSSION

The ploidy levels of the plants were inferred from the mean fluorescent intensity of PI-stained nuclei (Figure 1 and Table 2).

Table 2. The mean fluorescent intensity (MFI) and inferred ploidy levels of *Fragaria* species.

No.	Species and interspecific hybrids	No. of studied	MFI (range)	Ploidy level
1	*F. vesca* ssp. *vesca*	3	234 (231.3-237)	2x
2	*F. vesca* ssp. *americana*	2	231.3 (231-231.6)	2x
3	*F. viridis*	1	230.4	2x
4	*F. nubicola*	2	249 (246-252)	2x
5	*F. nipponica*	2	NA	2x[1]
6	*F. nilgerrensis*	1	NA	2x[1]
7	*F. daltoniana*	2	NA	2x[1]
8	*F. orientalis*	1	384	4x
10	*F. moschata*	1	614	6x
11	***F. iinumae*[1]**	**1**	**799**	**8x**
12	*F.* x *ananassa*	4	801 (799.6-803.3)	8x
13	*F. virginiana* ssp. *platypetala*	1	792.9	8x
14	*F. virginiana* ssp. *virginiana*	1	801	8x
15	*F. virginiana*	1	766	8x
16	*F. chiloensis* ssp. *pacifica*	1	766	8x
17	*F. chiloensis* ssp. *chiloensis*	1	756	8x
18	*F. chiloensis*	1	738	8x
19	*F.* x *ananassa* cul. Vivorosa	1	796	8x

[1]The plant labelled as *F. iinumae* was found to be octoploid based on flow cytometry analysis with an MFI of 799.

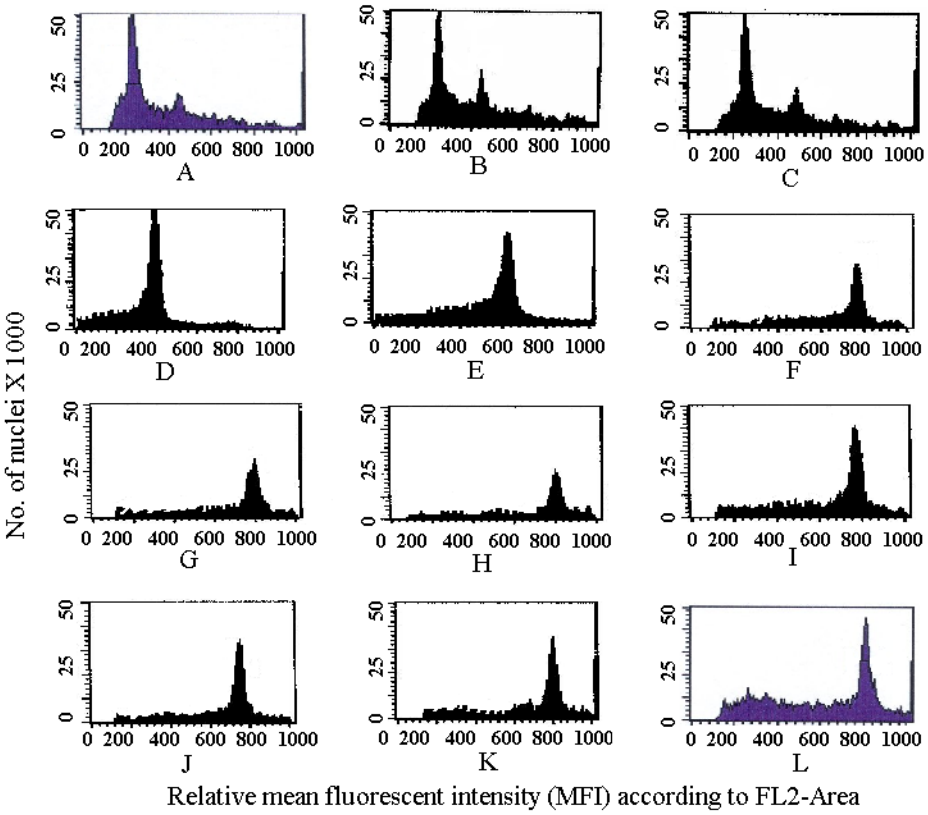

Figure 1. Flow-cytometric histograms showing mean fluorescent intensity (MFI) of *Fragaria* species. A) *F. vesca*, B) *F. nubicola*, C) *F. viridis*, D) *F. orientalis*, E) *F. moschata*. F) *F. virginiana* ssp. *virginiana*, G) *F. virginiana* ssp. *platypetala,* H) *F. chiloensis* ssp. *chiloensis,* I) *F. chiloensis* ssp. *pacifica*, J) *F.* x *ananassa* cul. *vivorosa*, K) *F.* x *ananassa* cul. *elsanta*, L) *F. iinumae*.

The plant labelled as *F. innumae* (NCGR accession number PI 551618 in NGR-Corvallis) was found to be octoploids as its MFI (799) was within the range obtained for otcoploid species. Since *F. innumae* is a diploid species, this indicates that this taxon was wrongly named by NCGR.

Four arbitrary RAPD primers produced 68 polymorphic, which were used for similarity comparison among species. The monomorphic bands were excluded from analysis (Figure 2). The genetic distances between pairs of taxa measured on the basis of Squared Euclidean Distance are shown in Table 3. At intra-subspecific level within *F. vesca* ssp. *vesca*, the genetic distances among different accessions of *F. vesca* ssp. *vesca* varied from 0 to 4 with the average value of 1.9, while the average distance between *F. vesca* ssp. *vesca* and *F. vesca* ssp. *vesca* f. *monophylla* was 1.7, indicating close genetic relatedness of these two taxa.

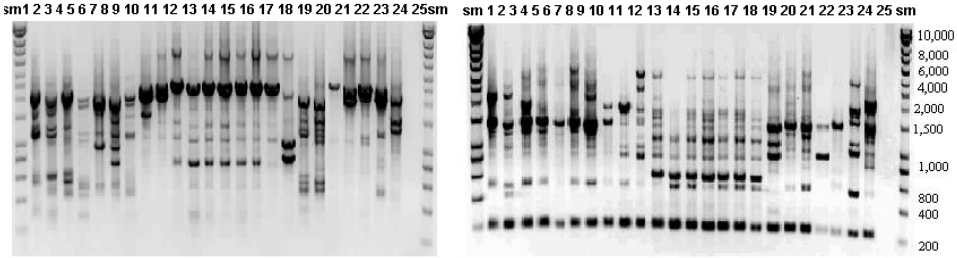

Figure 2. Samples of RAPD pattern of Fragaria species produced by primers D (A) and E (B). The lane numbers (1-24) correspond to the numbers in Table 1, and lanes of 25 are the negative control (sm= standard size markers=Hyper ladder I).

Table 3. Genetic distances matrix between pairs of species constructed from RAPD patterns in *Fragaria* species using Squared Euclidean Distance.

	Plant number according to Table 1																						
Plant No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	0																							
2	7	0																						
3	7	8	0																					
4	14	15	13	0																				
5	11	12	10	11	0																			
6	10	15	13	18	7	0																		
7	13	14	18	17	12	7	0																	
8	30	27	31	30	31	34	31	0																
9	35	32	36	35	36	39	34	7	0															
10	42	39	43	42	43	44	41	12	9	0														
11	40	37	41	40	41	42	39	12	13	4	0													
12	43	40	44	43	44	45	42	13	10	1	3	0												
13	43	40	44	43	44	45	42	13	10	1	3	0	0											
14	43	40	44	43	44	45	42	13	10	1	3	0	0	0										
15	43	40	44	43	44	45	42	13	10	1	3	0	0	0	0									
16	40	37	41	40	41	44	41	10	9	2	4	3	3	3	3	0								
17	36	33	37	30	37	40	35	16	15	18	22	19	19	19	19	18	0							
18	14	15	13	18	15	18	17	28	33	38	38	39	39	39	39	36	30	0						
19	32	31	33	26	31	34	31	16	23	24	24	25	25	25	25	22	12	24	0					
20	29	26	30	23	30	33	28	11	16	21	21	22	22	22	22	19	9	23	11	0				
21	31	28	32	25	32	35	30	11	16	21	21	22	22	22	22	19	9	27	9	4	0			
22	34	31	35	32	35	38	35	12	11	14	14	15	15	15	15	12	12	32	18	13	11	0		
23	28	27	29	24	29	32	31	18	23	26	22	25	25	25	25	24	18	28	20	15	17	18	0	
24	27	24	28	23	28	31	28	17	22	27	25	28	28	28	28	25	19	27	21	14	16	17	3	0

At inter-subspecific level, *F. vesca* ssp. *californica* and *F. vesca* ssp. *americana* were genetically more distant from *F. vesca ssp. vesca* with genetic distances of 10.3 and 12.3, respectively.

At inter-specific level, the distances between *F. vesca ssp. vesca* and the diploid species of *F. viridis*, *F. nubicola*, *F. daltoniana*, *F. nipponica* and *F. nilgerensis* were greater than those distances at intraspecific level within *F. vesca*, and ranged from 14.3 to 24.3. At inter-ploidy level, the greatest genetic distances were found to be between *F. vesca ssp. vesca* and the octoploids and varied from 39 to 44.3.

The species under study in the dendrogram based on Squared Euclidean Distance of RAPD data using clustering method of Between-Groups Linkage

were clustered into two groups (Figure 3). One cluster includes all octoploids and another cluster comprises of all diploids together with tetraploid and hexaploid species. All taxa belonging to *F. vesca* ssp. *vesca* were nested in one subcluster, while *F. vesca* ssp. *americana* and *F. vesca* ssp. *californica* in the second subcluster. The other five diploid species were nested in the third subcluster while polyploids *F. moschata* and *F. orientalis* formed the fourth subcluster, in which tetraploid and hexaploid are separated from diploids.

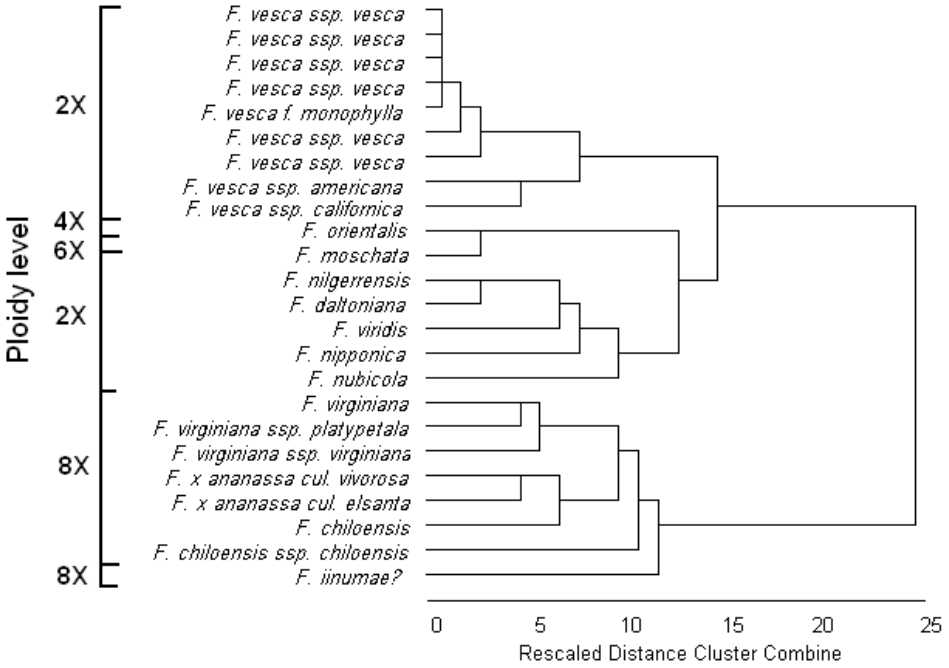

Figure 3. Dendrogram showing relationship among Fragaria species. The tree was constructed using cluster method of Between-Groups Linkage (SPSS version 13.2) on the basis of Squared Euclidean Distance calculated from RAPDs data.

All octoploid species nested in the second cluster alongside the plant labelled as *F. iinumae*. In this cluster, taxa belonging to *F. virginiana* formed a distinct subcluster, while those of *F. chiloensis* in another subcluster, while accessions of *F. x ananassa* gather in a separated subcluster between the subclusters of *F. virginiana* and *F. chiloensis*.

The results of the current study provided some information on the phylogeny relationship among species of *Fragaria*. These data clearly separated the genus species of *Fragaria* into two distinct lineages; one group includes all diploid species together with tetraploid *F. orientalis* and hexaploid *F. moschata,* while in the other cluster comprised of all octoploid species. The data provided a clear evidence for a close relationship among octoploids *F. virginiana*, *F. chiloensis* and *F.* x *ananassa*, and that the natural hybrid *F.* x *ananassa* has

originated from between the first two octoploids. This finding is consistent with data obtained from cpDNA (Harrison *et al.,* 1997a) and nuclear ITS data (Potter *et al.,* 2000). In addition, the current study also based on RAPDs also showed that there is a close relationship between *F. orientalis* and *F. moschata*, which has been previously supported by cpDNA data (Harrison *et al.,* 1997a) and nuclear ITS region (Potter *et al.,* 2000).

The results of the present work supports the current taxonomy of octoploid *Fragaria* at subspecies level by placing *F. virginiana* ssp. *platypetala* within species of *F. virginiana*, and disagrees with those results previously obtained from morphometric and RAPD (Harrison *et al.,* 1997b), and nuclear ITS/*trn* studies (Potter *et al.,* 2000), which have considered *F. virginiana* ssp. *platypetala* more closely related to *F. chiloensis*.

The RAPD study did not provide evidence for the ancestral origin of octoploids, neither did previous morphometric (Harrison *et al.,* 1977b) and ITS sequence data (Potter *et al.,* 2000), although chromosomal and cytological data have proposed *F. vesca* as the ancestor for all octoploids (Bringhurst and Khan, 1963; Senanayake and Bringhurst, 1967). This postulation has mostly been undermined by the occurrence of *F. vesca* as the only diploid species in the octoploids distribution areas in North America. Current RAPD data indicate that the octoploids are well defined group but, as the results cannot offer information regarding their origin, the ancestral origin of octoploids still remains unknown. However, according to Potter *et al*. (2000) the most closely related diploids to the octoploids *F. chiloensis* and *F. virginiana* are *F. vesca* and *F. nubicola*, but data from the present study do not support this relationship, rather suggests *F. nilgerrensis* as the closest diploid species to the octoploids.

The two wild octoploids *F. chiloensis* and *F. virginiana* alongside their natural interspecific hybrid *F. x ananassa* are distributed in the New World, but Staudt (1973) described a third wild octoploid species, *F. iturupensis*, from the Kuril Islands in East Asia. Based on this report, Harrison *et al*. (1997a) raised the possibility that the octoploids could have originated in Asia and then migrated to America across the Bering Strait, later evolved into two species (*F. virginiana* and *F. chiloensis*). Among the plant material used in the current study, a plant labelled by NCGR as *F. iinumae* (NCGR accession number PI 551618 in NGR-Corvallis) was found to be octoploid on the basis of MFI. In addition, this plant has occupied a position on the RAPD-based dendrogram implicating ancestral origin for the other octoploids. This taxon can not be diploid *F. iinumae*.

The RAPDs data disagree with Staudt (1989) in suggesting *F. nubicola* as the ancestral origin for *F. moschata*, rather propose that *F. vesca* and one of five diploids (*F. viridis*, *F. nubicola*, *F. nilgerensis*, *F. daltoniana* and *F. nipponica*) were most likely involved in the formation of hexaploid *F. moschata*. This is further supported by the fact that the distribution areas of *F. vesca* and *F. viridis* do overlap with that of *F. moschata* in Europe, and natural hybrid between these diploids (*F.* x *bifera*) has many times occurred throughout the area in Europe (Staudt *et al*., 2003). While the geographic distribution of *F. nubicola* is far away

from that of *F. moschata* since *F. nubicola* is limited in the Himalayas occurring from Kashmir to western China while *F. moschata* is found in Europe, Turkey and Central Russia (Tutin *et al*., 1968).

All subspecies of *F. vesca* strongly clustered into one group in this study and supports their classification at this taxonomic level. The nesting of *F. vesca* ssp. *vesca* f. *monophylla* within all other subspecies of *F. vesca* provided support for its taxonomic position suggested by Staudt (1962) as a forma of *F. vesca* ssp. *vesca*.

CONCLUSIONS

This study revealed some evolutionary and taxonomic aspects in the genus *Fragaria*. The results from the RAPDs-dendrogram suggest that polyploid formation in *Fragaria* has at least occurred two times: once resulting in tetraploid and hexaploid species in Eurasia and in the second event in America gave rise to the octoploids.

ACKNOWLEDGEMENTS

I am grateful of Dr K. Hummer (National Clonal Germplasm Repository, Corvallis, Oregon, USA) for providing a wide range of *Fragaria* materials.

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Houshang NOSRATI

FILOGENETSKI ODNOSI MEĐU VRSTAMA FRAGARIA ZASNOVANI NA RAPD

SAŽETAK

Rod *Fragaria* L. (jagoda, Rosaceae) sastoji se od 22 vrste različite ploidnosti. Evolucija ploidnosti kod *Fragaria* nije potpuno jasna. Filogenija *Fragaria* je ispitivana primjenom RAPD. Nivo ploidnost je izračunat iz sadržaja DNK i to protočnom citometrijom. Genetska distanca je izračunata upotrebom kvadrat-euklidskog rastojanja, a analiza nastalih klastera je urađena metodom međugrupnog povezivanja (Between-Groups Linkage). Četiri od deset ispitivanih prajmera proizvelo je 68 polimorfnih i reproduktivnih grupa. Najmanja genetička udaljenost (1.7) pronađena je između *F. vesca* ssp. *vesca* i *F. vesca* ssp. *vesca* f. *monophylla*, dok je razlika između *F. vesca* ssp. *vesca* I *F. vesca* ssp. *californica* i *F. vesca* ssp. *americana* iznosila 10.3 odnosno 12.3. Najveća udaljenost između *F. vesca* ssp. *vesca* i oktoploida iznosila su 39. - 44.3. Klaster analizom sve vrste su grupisane u dvije grupe: u jednom klasteru su svi oktoploidi, a drugom klasteru su diploidi zajedno sa tetraploidima i heksaploidima. Podaci su pokazali tijesnu vezu između *F. orientalis* i *F. moschata*, kao između oktoploida *F. virginiana*, *F. chiloensis* i *F. x ananassa*, i pretpostavku da je *F. x ananassa* hibrid između *F. virginiana*, *F. chiloensis.* Ovaj rad podržava postojeću taksonomiju oktoploida na nivou podvrsta, ali se ne slaže sa morfometrijskim i ITS/trn podacima, i ukazuje na to da da je kod *Fragaria* do poliplododnosti došlo dva puta: prvi put su proizvedeni tatraploidi i heksaploidi u Evroaziji, i drugi put oktoploidi u Americi.

Ključne riječi: *Fragaria*, jagoda, filogenetski odnosi, evolucija poliploida