Molecular analysis of strawberry cultivars using RAPD, AP–PCR and STS markers

Balogh, A.[1], Kiss, E.[1], Szőke, A.[1], Dénes, F.[2] and Heszky, L.[1]

[1]*Szent István University, Faculty of Agricultural and Environmental Sciences, Department of Genetics and Plant Breeding, 2103 Gödöllő, POB 303, Hungary*
e-mail: heszky@fau.gau.hu
[2]*Fertőd Fruit Production Research and Development Institute, H-9435 Sarród, Hungary*

Summary: Seventeen strawberry (*Fragaria x ananassa* Duch.) cultivars representing the national list of Hungary, were subjected to RAPD, AP–PCR and STS analysis. Of the 31 decamer and oligomer primers tested 26 primers produced polymorphic patterns. 45 polymorphic fragments were analysed, ranging between 200-2800 bp in size. Based on the data, similarity coefficients (Jaccard index and Simple matching coefficient) were calculated, and dendrograms were constructed using the unweighted pair group method of arithmetic averages (UPGMA). The dendrograms only partly reflect the known pedigree data. Specific RAPD markers were identified for cultivars F5, Pocahontas and Rabunda.

Key words: cultivar identification, *Fragaria x ananassa*, genetic distance

Introduction

The cultivated strawberry *Fragaria x ananassa* is a natural hybrid between two octoploid *Fragaria* species (x=7, 2n=56), *F. virginiana* Duch. and *F. chiloensis* (L.) Duch. (Hancock et al., 1996). Because the high number of commercial strawberry cultivars which are derived from controlled hybridizations mainly among other octoploid cultivars, there is a need for developing reliable molecular markers for cultivar identification. Estimation of genetic distance between cultivars is also a necessary tool for breeders to decide the potential parents for further improvement of a specific trait. Molecular markers, especially RAPD markers have been used for analysis of genetic stability in micropropagated strawberries *(Kumar* et al., 1999), for cultivar identification *(Congiu* et al., 2000), and for genetic distance assessment between cultivars *(Hancock* et al., 1994, *Landry* et al., 1997, *Degani* et al., 1998). These studies reported good agreement between RAPD data and pedigree information. Recently AFLP markers have been used for measuring genetic relationship *(Degani* et al., 2001) with the purpose to compare the pedigree information obtained from AFLP, RAPD and pedigree data. Correlation tests revealed that the AFLP-derived genetic similarity values showed insignificant correlation with the coefficients of coancestry values. A better correlation was observed between the RAPD-derived genetic similarity values and the coefficients of coancestry. RAPD markers were also used for linkage map construction of the diploid *Fragaria vesca* L. *(Davis & Yu,* 1997) and tagging disease resistance genes and SCAR marker developement in the cultivated strawberry *(Haymes* et al., 1997, 2000).

The objectives of this study were *a*: to identify polymorphic AP-PCR (arbitrarily primed polymerase chain reaction), RAPD (randomly amplified polymorphic DNA) and STS (sequence tagged site) markers useful for distinguishing the seventeen strawberry cultivars, representing the national list of Hungary, *aa*: to identify specific markers for the two cultivars (F5 and Kortes) bred in Hungary and *aaa*: to estimate the genetic relatedness of these cultivars using molecular markers.

Material and method

Plant material: The seventeen strawberry cultivars, evaluated in this study are listed in *Table 1.* Plant material was obtained from the strawberry germplasm collection of the Hungarian Fruit Production Research and Development Institute, Fertőd.

DNA extraction: Young, not fully expanded leaves were collected from 2–3 plants of each cultivar. To extract DNA, the protocol described by Aitchitt et al. (1993) was used, with the following modification: after grinding of 2–3 g leaves in liquid nitrogen, a washing buffer was added to the samples *(Mercado* et al., 1999). DNA concentrations were estimated by 1% agarose gel electrophoresis and ethidium bromide staining. The strawberry genomic DNA was diluted in sterile water to the proper concentrations for PCR reactions.

DNA amplification: For RAPD, AP-PCR and STS, reactions were performed in a volume of 25 μl in a Bio-Rad

(Fermentas). Gels were stained with ethidium bromide and Sybr® Gold (Molecular Probes) if it was neccessary, visualised under UV light and photographied using a Polaroid camera.

Data analysis of RAPD, AP-PCR and STS reactions, fragments were scored as present (1), or absent (0). A dendrogram was constructed based on the similarity matrix data by applying the unweighted pair-group method with arithmetic averages (UPGMA) cluster analysis using the SYN-TAX program package for PC Version 5.0 *(Podani,* 1993). For the estimation of genetic distances between cultivars the Simple matching coefficient *(Sokal & Michener,* 1958), and the Jaccard index *(Jaccard,* 1908) were used.

Results and discussion

RAPD reactions were performed with 28 decamer random primers, but only 25 primers generated reproducible patterns *(Table 2).* Four of the RAPD primers (OPA02, OPA11, OPAB03, UBC89), one of the AP–PCR primers (MGLAE) and one of the STS primers (*cyst* 1f / *cyst* 1r) generated monomorphic patterns. The number of polymorphic fragments obtained and taken into account, in case of 21 RAPD primers, 3 AP-PCR primers and 1 STS primer was 45 (1–4 fragments from each of the 26 primers). The molecular weight of the fragments was between 200 bp (UBC354, *adh* 1f / *adh* 1r) and 2800 bp (OPAB05, OPAB08), the number of generated fragments ranged between 1–12 for each primer. *Figure 2. (A and B)* showes polymorphic patterns obtained by RAPD primer OPE02, and AP–PCR primer WFRVC.

STS reactions with the primer pair designed for cystathionine-g-synthase gene *(Table 4),* based on the nucleotide sequence of diploid *Fragaria vesca* L. *(Marty* et al., 2000), amplifies a 540 bp region of the exons IV–V. In the studied cultivars we obtained this single PCR product of 540 bp, generating a monomorphic pattern *(Figure 2, C).*

The ADH primer pair *(Table 4)* amplifies a 460 bp region spanning introns II and III alcohol dehydrogenase gene *(Wolyn & Jelenkovic,* 1990) of the octoploid strawberry (*Fragaria x ananassa* Duch.). In our study this primer pair generated three PCR products of 200, 450 and 580 bp, resulting in a polymorphic pattern *(Figure 2, D).* This primer pair was used in linkage map construction of the diploid strawberry (*Fragaria vesca* L.), where it generated a single PCR product *(Davis & Yu,* 1997). Our study suggests that the *adh* gene should have several loci in the octoploid strawberry as it was supposed by *Wolyn & Jelenkovic,* (1990).

Based on the 45 polymorphic fragments generated in RAPD, AP–PCR and STS reactions, Jaccard indices and Simple matching coefficients were calculated, and dendrograms were constructed *(Figure 1).* The dendrograms were compared to the information available concerning the place of origin and the immediate parents of the cultivars shown in *Table 1 (Papp & Porpáczy,* 1999).

For RAPD pattern analysis the use of Jaccard index is more suitable, taking into consideration that RAPD and AP-PCR reactions generate dominant markers, and this index considers the polymorphic fragments in a higher proportion than other similarity coefficients do *(Link* et al., 1995). The Simple matching coefficient differs from the Jaccard index by taking into account not only the common but also the missing bands of the compared sample pairs *(Stiles* et al., 1993).

The dendrogram based on Jaccard index *(Figure 1, A)* contains two main clusters, both of them are subdivided into several subclusters.

In one of the main clusters cultivars Bogota, Kortes, Honeoye, Elsanta, Gerida, Gorella, Onebor are grouped together. Except Bogota and Kortes, the rest of the cultivars are closely related by their parentage, too *(Table 1).* Bogota and Kortes have no common parents with the mentioned

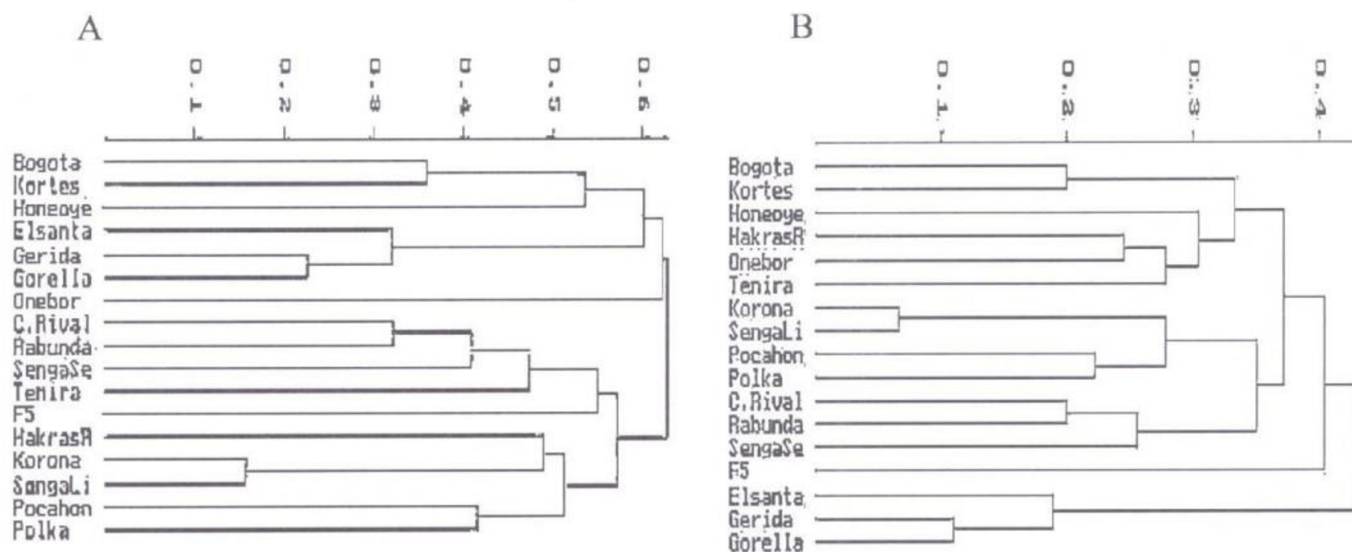

Figure 1 Dendrograms obtained with the cluster analysis of seventeen strawberry cultivars. Genetic distances obtained with Jaccard index (A), and Simple matching coefficient (B).

cultivars, though they cluster with them. In the other main cluster of this dendrogram, the cultivars did not group together in any obvious pattern. This cluster contains the cultivars C. Rival, Rabunda, Senga Sengana, Tenira, F5, Hakra's Romata, Korona, Senga Litessa, Pocahontas, Polka. Although Senga Sengana and Senga Litessa, or Rabunda and Tenira are closely related by their parentage *(Table 1)*, they are not directly clustered.

The dendrogram based on the Simple matching coefficient *(Fig. 1, B)* contains three main clusters, one of them grouping 13 cultivars in two subclusters, the other one contains only the cultivar F5, and the third one put together cultivars Elsanta, Gorella and Gerida. The main difference between the two dendrograms is that the above mentioned three cultivars in the second dendrogram (B: Simple matching coefficient) are not in the same cluster with the

cultivars Honeoye and Onebor, though they are closely related according to their parentage, and this is reflected by the dendrogram based on the Jaccard index.

Molecular based cluster analyses only partly reflect the genetic relationships expected by the known pedigree of cultivars. This fact may be due to the insufficient number of analysed markers *(Levi & Rowland,* 1997) or the characteristic of RAPD analyses which can be useful for distinguishing different varieties but do not always estimate the genetic relationships of cultivars *(Degani* et al., 1998).

Strawberry cultivar identification still relies on morphological characters of leaf, flower and fruit *(Dale,* 1996). Because of the high number of sometimes closely related cultivars this method of identification is often unsuitable. Molecular markers are fast and reliable tools of distinguishing cultivars, they even have forensic application *(Congiu* et al., 2000).

Another objective of this study was to find reliable and specific markers for strawberry cultivars. RAPD primer OPC08 generated a 500 bp fragment specific for cultivar F5, originated from Hungary, primer OPAB09 a fragment of 700 bp specific for cultivar Pocahontas, and with primer OPAB20 we have obtained a band of 350 bp characteristic for Rabunda, a day-neutral cultivar. In a further study our aim is to convert these RAPD products into SCAR markers for reliabile variety identification.

Acknowledgement

This research was supported by the Ministry of Education (FKFP 380 / 2000) and the Hungarian Scientific Research Fund (OTKA TS 040887).

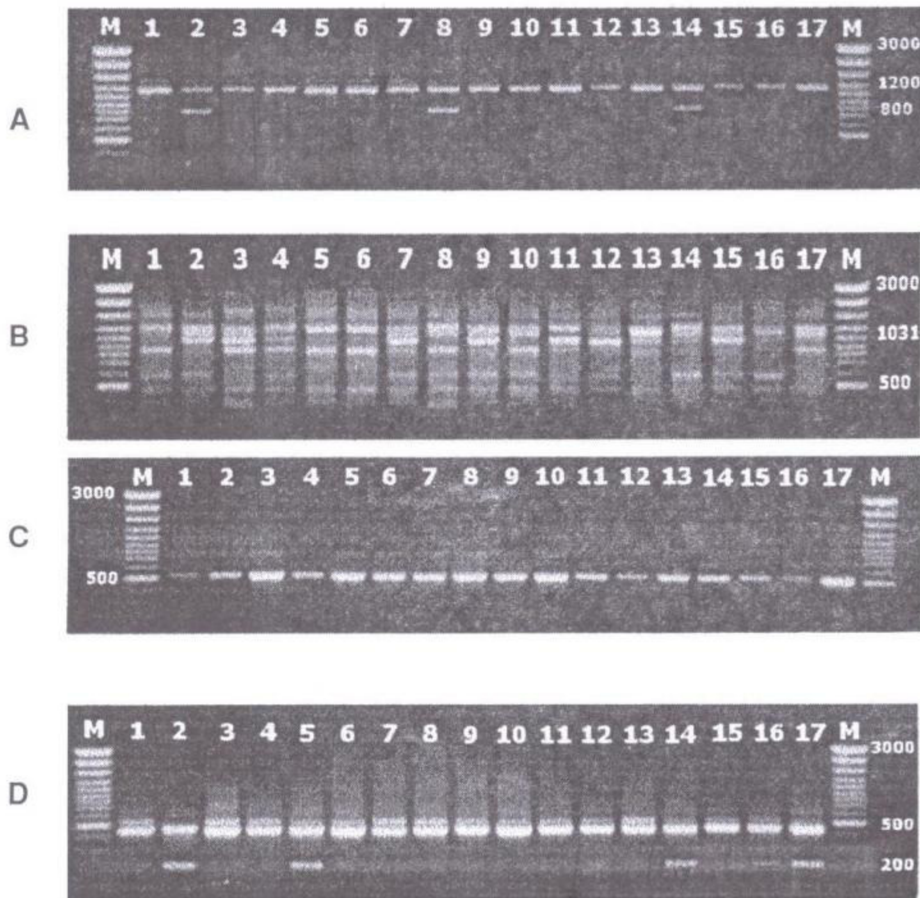

Figure 2 DNA profiles generated by primers: OPE02 (A), WFRV C (B), *cyst* 1f/ *cyst* 1r (C), *adh* 1f/ *adh*1r (D). M: 100bp ladder (Fermentas), 1–17: cultivars listed in *Table 1*.

Table 1 The cultivars used, their origin and immediate parents

Cultivar	Origin	Parents
1. Bogota	NL	Zaltbommel 53116 X Tago
2. Cambridge Rival	GB	Dorsett X Early Cambridge
3. Elsanta	NL	Gorella X Holyday
4. F5	HU	Redstar X Unknown
5. Gerida	CH	Elvira X Elsanta
6. Gorella	NL	Juspa X US 3763
7. Hakra's Romata	DE	Unknown
8. Honeoye	US	Vibrant X Holyday
9. Korona	NL	Tamella X Induca
10. Kortes	HU	Fertődi 602/16 X F602/2
11. Onebor	IT	Unknown X Gorella
12. Pocahontas	US	Tennesse Schipper X Midland
13. Polka	NL	Induca X Confitura, 5
14. Rabunda	NL	Red Gauntlet X Repita
15. Senga Litessa	DE	Markee X Unknown
16. Senga Sengana	DE	Markee X Sieger
17. Tenira	NL	Red Gauntlet X Gorella

iCycler thermal cycler. The reaction mixture contained 10–20 ng of individual strawberry genomic DNA as template, 5 picomoles of random primer in RAPD and AP-PCR or 2 x 2.5 picomols in STS, 2.5 μl of 10 x reaction buffer, 2 mM $MgCl_2$, 75 μM dNTPs and 1.2 units RedTaq polymerase (Sigma). The thermal cycler was programmed as followes: 2 minutes at 94 °C, followed by 40 times: 10 seconds at 94 °C, 30 seconds at the annealing temperature, 1 minute at 72 °C, the final cycle with 2 minutes at 72 °C. The annealing temperatures used for AP–PCR and STS primers are shown in *Table 3* and *Table 4*. The annealing temperature in RAPD reactions was 36 °C. RAPD primers were obtained from Operon Technology (Alameda, California), and the University of British Columbia (Vancouver, Canada; UBC89 and 354), AP–PCR primers DEIYA, WFRVC, MGLAE are primers specific for apple ACC-synthase (1-aminocyclopropane-1-carboxylate synthase) gene designed at our department, primer DX52 is the pair of DX51, tagging the high-molecular weight glutenin in wheat. The four AP–PCR and the STS primers for detecting strawberry alcohol dehydrogenase (ADH) gene *(Wolyn & Jelenkovic,* 1990, *Davis & Yu,* 1997) and cystathionine γ-synthase (CGS) gene of the wild strawberry *(Marty* et al., 2000) were synthetized by BRC of HAS (Dr. Sándor Bottka, Biological Research Center of the Hungarian Academy of Science, Szeged, Hungary). All PCR reactions were repeated twice, and only reproducible DNA bands were retained for analyses.

PCR products were subjected to electrophoresis on 1.2% TAE agarose gels along with a 100 bp DNA ladder

Table 2 Primers used in RAPD reactions and the number of polymorphic bands obtained

RAPD primer name	Nucleotid sequence (5'→3')	Number of polymorphic fragments	RAPD primer name	Nucleotid sequence (5'→3')	Number of polymorphic fragments
OPA02	TGCCGAGCTG	0	OPAI21	CACGCGAACC	2
OPA11	CAATCGCCGT	0	OPAL07	CCGTCCATCC	2
OPA18	AGGTGACCGT	3	OPAL20	AGGAGTCGGA	3
OPAB02	GGAAACCCCT	1	OPB07	GGTGACGCAG	2
OPAB03	TGGCGCACAC	0	OPC08	TGGACCGGTG	1
OPAB04	GGCACGCGTT	2	OPE02	GGTGCGGGAA	1
OPAB05	CCCGAAGCGA	2	OPX03	TGGCGCATGG	1
OPAB08	GTTACGGACC	2	OPX11	GGAGCCTCAG	1
OPAB09	GGGCGACTAC	2	OPV02	AGTCACTCCC	1
OPAB14	AAGTGCGACC	1	OPV07	GAAGCCAGCC	1
OPAB17	TCGCATCCAG	1	OPV15	CAGTGCCGGT	1
OPAB20	CTTCTCGGAC	4	UBC89	GGGGGCTTGG	0
			UBC354	CTAGAGGCCG	1

Table 3 Primers used in AP-PCR reactions, their annealing temperatures, and the number of polymorphic fragments obtained

AP–PCR primer name	Nucleotid sequence (5'→3')	Annealing temp.	Number of polymorphic fragments
DEIYA	GACGAGATCTACGC	40 °C	4
WFRVC	CAAACTCGGAACCA	43 °C	4
MGLAE	ATGGGTCTTGCAGAG	43 °C	0
DX52	GAAACCTGCTGCGGACAAG	55 °C	1

Table 4 Primers used in STS reactions, their annealing temperatures and the number of polymorphic fragments obtained

STS primer name	Nucleotid sequence (5'→3')	Annealing temp.	Number of polymorphic fragments
*cys*1f / cyst1r	CATATTGTGACAACAACAGAC CACAACAAGATCAGCCCCAAG	60 °C	0
adh 1f / *adh* 1r	CCAAGGTACACATTCTTTTTTTC CTCTCCACAATCCTGAATTTTAG	55 °C	1

References

Aitchitt, M., Ainsworth, C. C. & Thangavelu, M. (1993): A rapid and efficient method for the extraction of total DNA from mature leaves of the date palm (*Phoenix dactylifera* L.). Plant Molecular Biology Reporter 11: 317–319.

Congiu, L., Chicca, M., Cella, R., Rossi, R. & Bernacchia, G. (2000): The use of random amplified polymorphic strawberry varieties: a forensic application. Molecular Ecology 9 (2): 229–232.

Dale, A. (1996): A key and vegetative descriptions of thirty-two common strawberry varieties grown in North America. Advances in Strawberry Research 15: 1–12.

Davis, T. M. & Yu, H. (1997): A linkage map of the diploid strawberry, *Fragaria vesca*. Journal of Heredity 88: 215–221.

Degani, C., Rowland, L. J., Levi, A., Hortynski, J. A. & Galetta, G. J. (1998): DNA fingerprinting of strawberry (*Fragaria x ananassa*) cultivars using randomly amplified plymorphic DNA (RAPD) markers. Euphytica 102: 247–253.

Degani, C., Rowland, L. J., Saunders, J. A., Hokanson, S. C., Ogden, E. L., Golan-Goldirsh, A. & Galletta, G. J. (2001): A comparison of genetic relationship measures in strawberry (*Fragaria x ananassa* Duch.) based on AFLPs, RADPs, and pedigree data. Euphytica 117: 1–12.

Hancock, J. F., Callow, P. A. & Shaw, D. V. (1994): Randomly amplified polymorphic DNAs in the cultivated strawberry, *Fragaria x ananassa*. J. Amer. Soc. Hort. Sci.119(4): 862–864.

Hancock, J. F., Scott, D. H. & Lawrence, F. J. (1996): Strawberries. In: Janick J. & Moore J. N. (Eds.), Fruit Breeding: Vine and Small Fruits. vol II, pp. 419–470. John Wiley & Sones, Inc., New York.

Haymes, K. M., Henken, B., Davis, T. M. & Van de Weg, W. E. (1997): Identification of RAPD markers linked to a *Phytophthora fragariae* resistance. Theor Appl Genet 94: 1097–1101.

Haymes, K. M., Van de Weg, W. E., Arens, P., Maas, J. L., Vosman, B. & Den Nijs, A. P. M. (2000): Development of SCAR markers linked to a *Phytophthora fragariae* resistance gene and their assessment in European and North American strawberry genotypes. J. Amer. Soc. Hort. Sci. 125 (3): 330–339.

Jaccard, P. (1908): Nouvelles recherches sur la distribution florale. Bull Soc Vaud Sci Nat 44: 223–270.

Kumar, M. B., Barker, R. E. & Reed, B. M. (1999): Morphological and molecular analysis of genetic stability in micropropagated *Fragaria x ananassa* cv. Pocahontas. In Vitro Cellular & Developmental Biology-Plant 35 (3): 254-258.

Landry, B. S., Rongqi, L. & Khanizadeh, S. (1997): Classification of 75 strawberry cultivars and breeding lines using RAPD markers. Acta Hort 439: 101–105.

Levi, A. & Rowland, L. J. (1997): Identifying blueberry cultivars and evaluating their genetic relationships using randomly amplified polymorphic DNA (RAPD) and simple sequence repeat-(SSR)-anchored primers. J Amer Soc Hort 122: 74–78.

Link, W., Dixkens, C., Singh, M., Schwall, M. & Melchinger, A. E. (1995): Genetic diversity in European and Mediterranean faba bean germplasm revealed by RAPD markers. Theor Appl Genet 90: 27–32.

Marty, I., Douat, C., Tichit, T., Jungsup, K., Leustek, T. & Albagnac, G. (2000): The cystathionine-γ-synthase gene involved in methionine biosynthesis is highly expressed and auxin-repressed during wild strawberry (*Fragaria vesca* L.) fruit ripening. Theor Appl Genet 100: 1129–1136.

Mercado, J. A, Mansouri, I. El, Jiménez-Bermúdez, S., Pliego-Alfaro, F. & Quesada, M. A. (1999): A convenient protocol for extraction and purification of DNA from *Fragaria*. Society for In Vitro Biology 5: 152–153.

Podani, J. (1993): Syn-Tax Version 5.0: Computer programs for multivariate data.

Papp J. & Porpáczy A. (1999): Szamóca, málna. Bogyósgyümölcsűek I., Mezőgazda Kiadó, Budapest, pp. 231.

Sokal, R. R. & Michener, C. D. (1958): A statistical method for evaluating systematic relationships. Univ Kansas Sci Bull 38: 1409–1438.

Stiles, J. I., Lemme, C., Sondur, S., Morshidi, M.B. & Manshardt, R. (1993): Using randomly amplified polymorphic DNA for evaluating genetic relationships among papaya cultivars. Theor Appl Genet: 85: 697–701.

Wolyn, D. J. & Jelenkovic G. (1990): Nucleotid sequence of an alcohol dehydrogenase gene in octoploid strawberry (*Fragaria x ananassa* Duch.). Plant Mol Biol 14: 855–857.