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THE APPLICATION OF RAPD MARKERS FOR GENETIC STUDIES ON *FRAGARIA X ANANASSA* DUCH. (STRAWBERRY) WITH DUE CONSIDERATION FOR PERMANENT FLOWERING

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ABSTRACT

The level of genetic diversity between 16 cultivars of strawberry (*Fragaria x ananassa* Duch.) and the wild species of *Fragaria virginiana* Duch. was studied on the basis of the analysis of their DNA (RAPD reaction – randomly amplified polymorphic DNA). Six 10-nucleotide primers generated jointly 354 bands, of which 94.8% were polymorphic and 5.2% monomorphic. The analysis of interrelations was carried out on the basis of Dice similarity matrix. Presentation of relationship between the analysed genotypes on the dendrogram obtained by means of the unweighted pair-group method with arithmetic average (UPGMA).

An attempt to identify molecular markers linked to day-neutral trait in the strawberry was carried out. The bulked segregant analysis (BSA) was performed to find differences between the two DNA samples obtained from the segregating population F_1 that was generated from the singular crossing of photoperiodically sensitive and insensitive cultivars helping identify a RAPD marker, that is probably responsible for the permanent fruit bearing of strawberries.

Key words: bulked segregant analysis (BSA), photoperiodism, Fragaria x ananassa, genetic similarity, RAPD markers

INTRODUCTION

The genetic enhancement of cultivated strawberries dates back to the mid-18th century. In the last five decades considerable progress has been made in breeding programmes. It is now estimated that there are approximately 500 commercially grown cultivars of strawberries throughout the world [8].

In recent years genetic markers associated with usable properties have proved particularly useful in plant breeding. Genetic markers enable observation of regrouping in cruciferous genomes and permanent and relatively quick analysis of the segregation of alleles, which facilitates the selection of mixed species [12].

So far only few isoenzymatic markers have been used in the identification of variants and in evaluation of the genetic diversity of strawberries. Apparently, the studies on isoenzymes are not highly effective. Arulsekar et al. [2] and Bringhurst et al. [4] managed to distinguish only 60% of genotypes (14 out of 22) by analysing three enzymes (phosphoglucoisomerase – PGI, leucine aminopeptidase – LAP, phospohoglucomutase – PGM).

The Eighties brought about the development of a method of DNA analysis based on the restriction fragment length polymorphism (RFLP) [3]. The restrictive analysis has been widely applied in horticultural plants, e.g. genetic mapping, designation of selected genes, verification of origin, identification of variants and determination of genetic diversity.

RFLP procedures are rather labour intensive: they include the construction of genome DNA or cDNA inventories and require the use of radioactive isotopes. Soon, the polymerase chain reaction (PCR) [19] gave rise to the development of other PCR related methods that made it possible to amplify the fragments of DNA unknown sequences. One of the most commonly used methods (developed by Williams et al. in 1990 [20]) is the random amplified polymorphic DNA (RAPD).

The analysis of polymorphism by means of this method does not require preliminary information concerning the DNA sequence under analysis. During the reaction, the sequences of many loci of the genome are amplified. The products of the RAPD reaction are segregated according to Mendel models; therefore, they can be successfully used as genetic markers. Michelmore et al. [14] have described the method of quick identification of the DNA sequences that are associated with the well known genes in plants. Their bulk segregant analysis allows for the comparison of two clustered DNA samples obtained from the single crossed population. Each clustered sample contained a mixture of DNA singles identical in terms of the analysed property or gene but retaining a random genetic composition.

The purpose of the study was to analyse the level of genetic diversity in many strawberry cultivars through the analysis of their DNA (reaction PCR-RAPD). An attempt was also made to find molecular markers (RAPD) connected with photo-insensibility. The markers obtained can be used in the preliminary selection of the seedling material in accordance with the required criteria. Such a selection is more credible, since it is performed on the DNA level and not on the basis of phenotypic symptoms that can be easily modified by the conditions of the environment.

MATERIALS AND METHODS

Plant material. Studies were carried out on 16 strawberry cultivars and on *Fragaria virginiana* Duch. Table 1 presents the origin of the analysed strawberry cultivars.

Table 1. Parents and country of origin of strawberry cultivars studied

Cultivar name	Breeding parents	Place of origin
Evita	Chandler × (Brighton × Gorella)	England
Mara des Bois	Gentox Ostara × (Redgauntlet × Korona)	France
Selva	Brighton × (Tufts × Pajaro)	California, USA
Calypso	Rapella × Selva	England
Geneva	(Steamliner × Fairfax) × Red Rich	New York, USA
Irvine	Douglas × Muir	California, USA
Ostara	Redgauntlet × Macherauch's Dauerente	Holland
Rapella	Tioga × Rabunda	Holland
Tango	Rapella × Selva	England

Table 1. cont.

1	2	3
Dukat	Koralowa 100 × Gorella	ISiK Skierniewice, Poland
Elsanta	Gorella × Holiday	Holland
Pandora	(Von Humboldt × Redstar) × Merton Dawn	England
Paula	Senga Tigajga × Merton Dawn	AR Lublin, Poland
Plena	Senga Sengana × Merton Dawn	AR Lublin, Poland
Teresa	Redgauntlet S_1 × Senga Sengana S_1	AR Lublin, Poland
Senga Sengana	Markee × Sieger	Germany

DNA extraction. The isolation of total cellular DNA was performed on the basis of a procedure designed by Doyle and Doyle [7] as modified by Rowland and Nguyen [18].

The concentration of DNA in the solution was measured on Gene Quandt spetrophotometer (Pharmacia). Subsequently all samples were dissolved with a solution of RNA-ase (10 µg/ml stock) to working concentration of 40 ng/µl and incubated for 1 hour at 37°C. The samples so prepared were kept at 0-4°C, and the concentrated DNA was kept at -20°C.

DNA amplification conditions and gel electrophoresis. The PCR – RAPD reaction was performed in a thermal cycler (Perkin-Elmer DNA Thermal Cycler 480) in Eppendorf test tubes (Sigma-Aldrich) of 500 µl each. The 25 µl of the reaction mixture was made up of the following: 18.3 µl dd H_2O; 2.5 µl 10× buffer for PCR (Finnzymes Oy); 2.0 µl dNTP MIX (Sigma-Aldrich); 1.0 µl 10-nucleotide primer with the concentration of 5 µM (DNA – Gdańsk); 0.2 µl (1.0 U) Taq DNA Polymerase recombinant (Finnzymes Oy) and 1.0 µl of genome DNA (40 ng/µl). 10× buffer for PCR contained: 100 mM Tris HCL, pH 8.8 in 25°C; 15 mM Mg Cl_2; 500 mM KCL and 1.0% Triton X-100. The mixture of dNTPs comprised 2.5 mM of each of the deoxynucleotides.

Prior to the thermal reaction, the thermal cycler was preheated to 92°C, and the tubes were placed inside. The first stage consisted in a preliminary denaturation of DNA at 92°C for 1 minute followed by 45 cycles of the reaction: at 92°C for 1 minute, at 36°C for 2 minutes and at 72°C for 2 minutes with final incubation at 72°C for 7 minutes.

The products obtained in the reaction (24 µl) and Low DNA Mass Ladder markers (Gibco BRL) were separated on the 1.5% agarose containing 0.01% of EtBr in 1× concentrated TBE buffer (89 mM Tris-borate; 2.5 mM EDTA) at 80 V for approximately 2.5 hours. The patters were visualised on a transiluminator and photographed using Polaroid film in order to document the results.

Data analysis. The pictures of the gel were scanned, and by means of Scion Image Beta 2 (Wayne Rasband, National Institutes of Health, USA, 1997) the co-ordinates of bands were measured in pixels. The results obtained were analysed using Excel 7.0 to obtain a matrix of binary codes. The presence or absence of a band was treated as a singular feature to which relevant values of 1 and 0 were attributed.
Genetic similarities were estimated using the Dice coefficient of similarity as in Nei & Li [15]:

$$\frac{2 \cdot n11}{2 \cdot n11 + n01 + n10}$$

where n11 designates the number of common bands for two compared genotypes, n10 – cases where the bands were visible only in the first genotype, and n01 – when they were visible in the other genotype only [6].
The obtained similarity indices (SI) were then used to estimate mutual distances (DI), by employing the following formula: DI = 1 – SI.

Cluster analysis was carried out on similarity estimates using the unweighted pair-group method with arithmetic average (UPGMA) using NT-SYS-PC [17]. The results were then represented as dendogram. In order to determine the accuracy with which the genotype system presented on the dendogram demonstrates genetic similarities between the objects Mantel matrix-correspondence test [13] was performed.

Dissimilarity indices (DI) between the analysed objects are presented in Table 3.

Bulked segregant analysis (BSA). The analysis of clustered segregants was designed to demonstrate differences between the two DNA samples obtained from the segregating population F_1 and originating from the singular crossing of photo-sensitive and insensitive cultivars.

The analytical material collected in the summer of 1999 was subjected to DNA extraction (in accordance with the afore-mentioned procedure) from the parent plants and from 41 samples of the F_1 generation that demonstrated diversification in terms of photoperiodical sensitivity. 2.5 µg of DNA from 20 June bearing plants of the F_1 population (first bulk) was mixed, as just as the DNA from 21 everbearing plants (second bulk), classified as such according to two year field observations. The markers were identified in two mixtures, composed of the DNA from plants that differed in their reaction to the day length.

Since the analysis of the F_2 population was not performed, the second test comprised dominant homozygotes and heterozygotes that cannot be distinguished phenotypically on the F_1 level. During the analysis 40 ng of combined DNA was used as a RAPD matrix.

RESULTS AND DISCUSSION

During the study sixty 10-nucleotide, randomly selected primers containing over 50% of G + C bases (DNA Gdańsk II s.c.). were used. None of them contained palindromic sequences. The use of 23 primers did not yield any amplification products. Only 10 out of the remaining 37 primers yielded from 1 to 5 significant polymorphic fragments as a result of amplification. A product of amplification was considered significant when it was sufficiently intensive and different in size from the adjacent fragments [5]. Banding patterns from 6 primers were used in the analysis of relationship (Table 2).

Table 2. Random primers used for the detection of polymorphism. Primer names are according to manufacturer's (DNA – Gdańsk) identyfication system

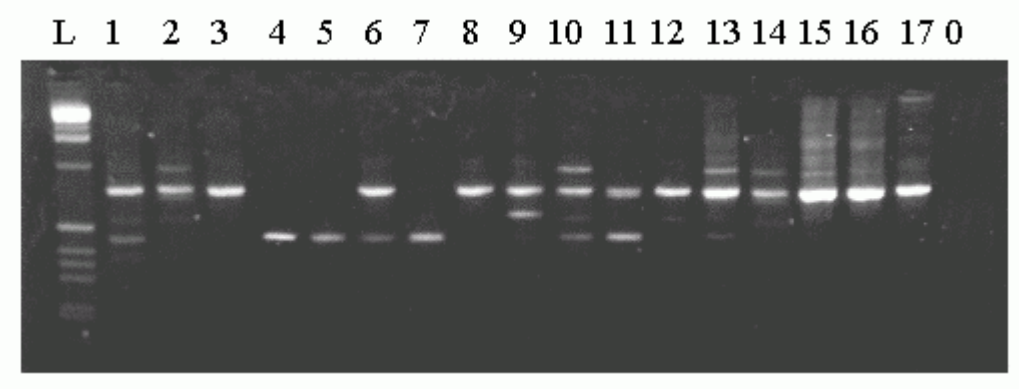
No.	Primer name	Nucleotide sequence (5' → 3')	Average number of fragments amplified for a single object	Size of amplification products (bp)	
				min.	max.
1	G 01	GGGAATTCGG	5.76	295	1174
2	G 02	TGCTGCAGGT	4.05	366	1687
3	D16	AGGGCGTAAG	2.41	654	2192
4	T 04	GTCCTCAACG	1.82	454	1377
5	J 19	GGACACCACT	4.47	329	963
6	G 10	CCGATATCCC	2.29	155	1034

Using the method described, no bands were observed in the control band (no DNA) and the reproducible results were obtained on the level of 90%. Repeatability was estimated as a coefficient of the number of common bands in both repeat tests to the total number of products observed in the first or second test multiplied by 100.

In order to obtain a matrix of binary codes, all possible combinations of genotypes and primer were analysed. The lengths of the amplification products were calculated using the 1-kb DNA ladder (Low DNA Mass Ladder, Gibco BRL).

Genetic diversity estimation. The six primers used in the analysis generated jointly 354 bands 94.8% of which were polymorphic and 5.2% were monomorphic. Figure 1 presents band patterns of DNA amplification products obtained by using T 04 primer.

Figure 1. Amplified DNA fragments from 16 strawberry genotypes: 'Ostara' (1), 'Calypso' (2), 'Evita' (4), 'Paula' (5), 'Pandora' (6), 'Plena' (7), 'Mara des Bois' (8), 'Dukat' (9), 'Selva' (10), 'Geneva' (11), 'Tango' (12), 'Senga Sengana' (13), 'Teresa' (14), 'Elsanta' (15), 'Rapella' (16), 'Irvine' (17) and Fragaria virginiana (3) using primer T 04. Molecular weight standards (1 kb ladder) are shown in the far left lane (L)



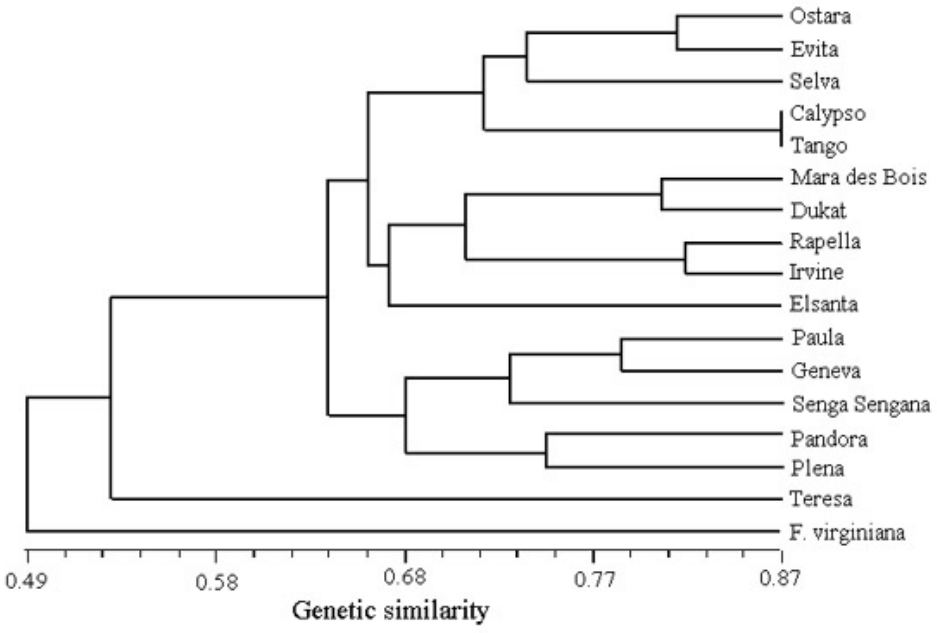
Graham et al. [10] analysed genetic similarities between 8 cultivars of strawberry. Using 10 primers for their analysis, they obtained 116 bands, 79 (68%) of which were polymorphic and 37 (32%) were monomorphic. These results were used to calculate genetic distances [15] and to prepare a dendrogram (using the UPGMA method). The similarity between the analysed variants ranged from 62 to 89% indicating their close relation.

In this study, the degree of similarity between 16 strawberry cultivars ranged from 53 to 87%. Similar results were obtained for other horticultural plants. Graham et al. [9] reported the similarity between the European variant of raspberry (*Rubus idaeus*) at 48-87% and blackcurrant variants (*Ribes nigrum*) at 24 to 77% [11]. The results obtained confirm the usefulness and applicability of the RAPD method in determining genetic similarity.

2 out of 8 strawberry cultivars analysed by Graham et al. [10], i.e. ‘Elsanta’ and ‘Evita’, were subject of our research. Genetic similarity between these cultivars was 72, vs. 83% obtained by Graham et al. [10].

The dendrogram presented in Figure 2 indicates that *F. virginiana* differs considerably from all analysed cultivars of *F.* x *ananassa.* This is confirmed by the length of the leading branches of the said species. Dice’s similarity index (SI) for the two cultivars is 0.49. The high value of the genetic distance (DI = 0.51) is in line with the expectations, since the differences between the taxons should increase along with the phylogenetic distance.

Figure 2. Dendrogram of strawberry cultivars based on RAPD data



The analysis of the data presented in Table 3 indicates that the genetic distance between ‘Calypso’ and ‘Tango’ is the smallest (DI = 0.13). This can be attributed to the fact that these cultivars have been selected from sister seedlings that originate from the crossing of ‘Rapella’ and ‘Selva’. Due to the marginal genetic distance (DI = 0.28), ‘Selva’, one of the parental forms, was marked on the dendrogram beside ‘Calypso’ and ‘Tango’ cultivars. The other parental form – ‘Rapella’, with the distance index at DI = 0.36, was not included in the group. ‘Evita’ and ‘Selva’ cultivars, which share the ancestor (‘Brighton’), were clustered together (DI = 0.32).

Table 3. Dissimilarity values between 16 strawberry cultivars: 'Ostara' (1), 'Calypso' (2), 'Evita' (4), 'Paula' (5), 'Pandora' (6), 'Plena' (7), 'Mara des Bois' (8), 'Dukat' (9), 'Selva' (10), 'Geneva' (11), 'Tango' (12), 'Senga Sengana' (13), 'Teresa' (14), 'Elsanta' (15), 'Rapella' (16), 'Irvine' (17) and Fragaria virginiana (3)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	0.000																
2	0.304	0.000															
3	0.476	0.524	0.000														
4	0.184	0.306	0.511	0.000													
5	0.421	0.368	0.647	0.415	0.000												
6	0.273	0.318	0.300	0.319	0.333	0.000											
7	0.381	0.381	0.579	0.467	0.294	0.250	0.000										
8	0.277	0.319	0.488	0.240	0.385	0.244	0.349	0.000									
9	0.348	0.391	0.524	0.347	0.421	0.273	0.381	0.192	0.000								
10	0.200	0.320	0.478	0.321	0.476	0.292	0.478	0.333	0.320	0.000							
11	0.317	0.317	0.568	0.364	0.212	0.282	0.351	0.333	0.317	0.378	0.000						
12	0.217	0.130	0.524	0.265	0.421	0.318	0.381	0.277	0.348	0.280	0.366	0.000					
13	0.381	0.333	0.579	0.422	0.294	0.300	0.368	0.349	0.333	0.348	0.243	0.381	0.000				
14	0.476	0.571	0.684	0.467	0.353	0.400	0.474	0.488	0.476	0.478	0.405	0.571	0.368	0.000			
15	0.319	0.362	0.488	0.280	0.487	0.422	0.488	0.333	0.319	0.373	0.333	0.362	0.395	0.535	0.000		
16	0.395	0.349	0.436	0.391	0.257	0.317	0.436	0.318	0.349	0.362	0.316	0.395	0.282	0.487	0.318	0.000	
17	0.333	0.333	0.421	0.378	0.294	0.300	0.368	0.209	0.286	0.348	0.297	0.333	0.368	0.526	0.349	0.180	0.000

‘Pandora’, ‘Plena’ and ‘Paula’ that originate from ‘Merton Dawn’ were grouped together. The genetic distance between ‘Plena’ and ‘Senga Sengana’, the other parental form, is 0.36. ‘Elsanta’ and ‘Dukat’ cultivars, originating from ‘Gorella’, were grouped in the same category. With the average distance at 0.49, ‘Teresa’ proved to be the most detached cultivar of them all.

The degree of accuracy with which the genotypes set presented on the dendrogram reflects genetic similarities between the objects was determined. For this purpose the Mantel matrix-correspondence test was applied by means of NT-SYS [17]. The correlation coefficient (r) was 0.81, i.e. the average value of the 0.80-0.90 bracket. This testifies to the correct grouping of the analysed genotypes.

The research conducted indicate the need to determine genetic diversity of parental forms in strawberry breeding programmes and a more effective use of unexploited or unrelated germplasms, that come from other geographical regions. The application of the RAPD method in the studies on strawberries facilitates a phylogenetic analysis and the need to seek specific links between markers and genes that are responsible for the vital properties of the plants such as their insensitivity to the length of the day.

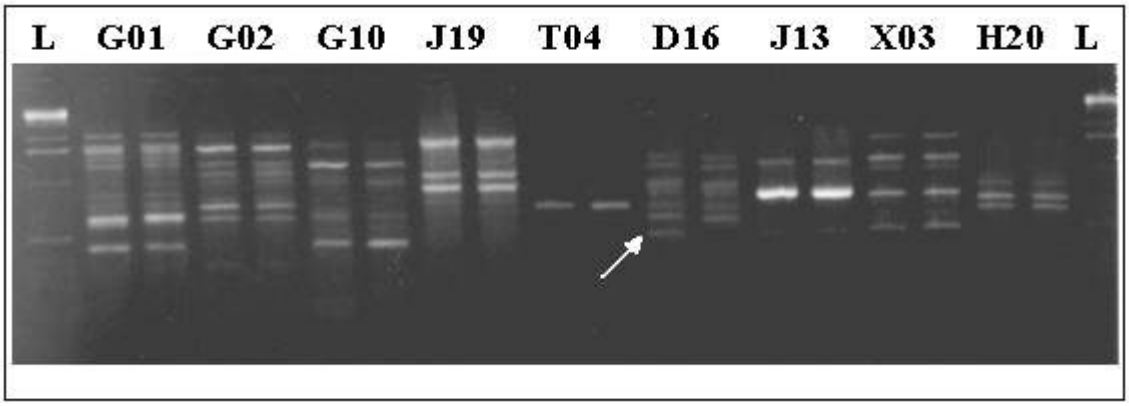
Identification of genetic markers correlated with photoperiodic insensitivity. The attempt to identify molecular markers connected with photoperiodic insensitivity in strawberries was a two stage venture.

During the first stage of the experiment, 60 randomly selected RAPD primers were analysed on two mixed DNA samples isolated from descending plants. Afterwards, 18 different primers were used, which distinguished two pools. The differences between them were confirmed in two repeat tests. Markers (bands) generated by the analysed primers and responsible for the variation differences were considered as likely markers of photoperiodic insensitivity. As a result of the first part of performed tests, four polymorphic markers were identified.

The second stage of the experiment was designed to determine the diversification – photoperiodic sensitivity/insensitivity – demonstrated in progeny is also present in the parental forms. Similar to RAPD markers, the trait of permanent fruit bearing in strawberry is a dominant feature [1, 16].

It turned out that only one marker, size 654 base pairs, generated by primer D 16 occurred in one of the parental forms (DN form) and in the second test with the corresponding reaction to the length of the day. Figure 3 presents D 16 marker, size 654 bp, which distinguishes the two bulks.

Figure 3. Identification of mixed DNA samples from June bearing and everbearing plants by using 9 primers. Arrows indicate marker D 16 size 654 bp, which distinguishes the two bulks



The studies conducted are of a preliminary nature. The next stage of research will involve preparing a map of interrelations within the strawberry genome, with due consideration for the genes responsible for photoperiodic insensitivity. Therefore, it is essential to test a greater number of primers (several hundred primers) in order to determine a grater number of markers. Afterwards, on the basis of the RAPD reaction data and by using JOINMAP computer software, interrelations between markers and target genes will be established. Only those markers that demonstrate strict correlation (<10 cM) with a given feature will be used in the process of selection.

The identification of sequences through the application of markers shall be carried out by means of available analytical methods. The presented research indicate that the BSA method based on the RAPD technique may be applied to identify molecular markers clustered with various dominant genes. This will facilitate the designing of modern selection methods that rely on testing young seedlings. The identification and application of markers in creative breeding of strawberry will shorten the time necessary to obtain new and valuable cultivars.

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