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Estimation of genetic divergence within androgenic regenerants of *Capsicum annuum* L. ATZ1 × *C. frutescens* L. F_1 plants using random amplified polymorphic DNA markers

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Abstract

To prove the androgenic origin of the regenerants obtained from the anther cultures of interspecific F_1 *C. annuum* L. ATZ1 × *C. frutescens* L.) hybrid, Random Amplified Polymorphic DNA (RAPD) method was applied. Thirty decamer primers were used in the experiment. The size of the amplified products ranged from 138 to 2369 bp, and their number from 3 to 25 per primer. About 274 loci, 47 of which were polymorphic, were analyzed. The results proved the hybrid status of anther donor plants detected by 20 of the applied primers. Of the 25 analyzed androgenic regenerants, 18 were diploids. The RAPD reactions confirmed their polymorphism in comparison with that of the F_1 hybrid generation, which excluded their origin from somatic cells of the anthers. Moreover, the results demonstrated the existence of a genetic variation among the obtained haploids, which illustrated genetic diversity of the microspores developing in hybrid anther cultures. The results of the experiments proved the utility of the RAPD method in detecting polymorphisms between closely related pepper plants, proving at the same time the effectiveness of androgenesis, regeneration of haploid plants, and spontaneously doubled haploids (DH lines).

Keywords: RAPD, PCR, hybrid, pepper, DH line, androgenesis

Introduction

Pepper (*Capsicum annuum* L.) is a plant of a high economic importance, cultivated and consumed all around the world. The wide usage of pepper fruits creates a demand for new modifications with additional and better qualities, thus providing resources satisfying all expectations of the industry. Breeding of *Capsicum* spp. focuses mostly on increasing the participation of desirable chemical compounds and improving the immunity of the plants to pathogens. A higher agronomic potential of pepper genotypes in terms of the said features is possible to be achieved, thanks to, for example, the application of interspecific hybridization and biotechnological breeding methods. The androgenic induction of embryos in *in vitro* anther cultures is a proven method in the case of *Capsicum* hybrids received in the process of inter- and intraspecific crossings. The effectiveness of this process and the proportion of haploids and diploids among regenerants depend mostly on the genotype of the mother plant (Irikova et al., 2011; Olszewska et al., 2014). For some genotypes, diploid plants constitute the majority of the regenerants received. The application of doubled haploid lines (DH lines) requires an effective identification method to exclude the possibility of regeneration from somatic tissues. The conventional method of identification of the pepper breeding material is based on the description of the morphological features. This method requires the collection of a large amount of data, which is time-consuming, and requires much work, experience, and money. For this reason, attempts have been made to assess the genetic varieties on the basis of the diversity among isozymes (Munyon et al., 1989; Dolcet-Sanjuan et al., 1997). Such variability is relatively low in breeding pepper; therefore, a biochemical ana-

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lysis can only be applied for interspecific hybrids of *Capsicum* (Olszewska et al., 2011). In the case of closely related genotypes, however, techniques enabling the detection of differences in DNA sequences are recommended (Mongkolporn et al., 2004; Paran et al., 2004; Ince et al., 2010). The effectiveness of methods basing on a polymerase chain reaction (PCR) in detecting polymorphisms in breeding forms of pepper has been confirmed and among them Random Amplified Polymorphic DNA (RAPD) was found to be the most promising one (Lefebvre et al., 2001, Jang et al., 2004, Sitthiwong et al., 2005; Sugita et al., 2005). The RAPD procedure is simple and fast, requires low quantities of DNA, and is easily automated. In addition, the RAPD technique does not require the use of radioactively labeled probes and can be applied without any former knowledge of genic sequences. In this reaction, single decamer primers of known but random sequence are typically used. Such a primer hybridizes with several priming sites on the complementary regions scattered around the tested genome. Therefore, polymorphism results from the number of complementary regions present in the genome (if these priming sites are within an amplifiable distance of each other) and is observed as the presence or absence of the amplified fragments visualized after the electrophoretic analysis of the PCR reaction products. In the presented research, the RAPD method was applied to prove the hybrid status of the androgenesis F_1 (*C. annuum* L. ATZ1 × *C. frutescens* L.) donor plants to verify the microsporic origin of diploid regenerants, and to assess the genetic variation in all the obtained androgenic regenerants.

Material and methods

Plant material

The research material consisted of F_1 (*C. annuum* L. ATZ1 × *C. frutescens* L.) hybrid, its parental forms: breeding *C. annuum* L. ATZ1 line and *C. frutescens* L., and 25 androgenic regenerants obtained in *in vitro* anther culture of F_1 hybrid. All material originated from the collection maintained by the Department of Plant Genetics, Physiology, and Biotechnology, the University of Sciences and Technology, Bydgoszcz, Poland. Anther donor plants were grown in unheated foil tents, following agrotechnical practices typical for annual pepper. Plants were not treated with pesticides or herbicides until the anther-sampling period was over.

In vitro cultures

Flower buds for anther isolation were harvested from healthy plants when the crown petals equaled or were slightly longer than the calyx sepals. Anthers cultures conditions maintained in the experiment were as described in the protocol by Dumas de Vaulx and coworkers (1981). All the culture media (CP, R1, and V3) used in this study were prepared as described by Chambonnet (1988). The modifications of the original protocol concerned the incubation time of the anthers on a CP medium for 14 days and the application of 0.3 mg/l kinetin in an R1 regeneration medium. The emerging embryos were transferred onto a V3 medium without growth regulators. The regenerated plants were thereafter acclimatized in a greenhouse. Androgenic regenerants were numbered from 1 to 25. The ploidy level of the plants was determined with the use of flow cytometry as described in Sliwinska (2003). In a cytometric assessment, the haploids were marked with the letter H and diploids with the letter D.

DNA isolation and RAPD analysis

All genomic DNA samples from a hybrid plant, the parents, and regenerants were extracted from 100 mg of healthy and the youngest leaves using GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich). The quality and purity of the extracted DNA probes were tested electrophoretically in 1% agarose gels stained with ethidium bromide and measured by spectorophotometry. Before the analysis, the DNA solutions were stored at −20°C. Thirty decamer primers were used in this study (Table 1). The RAPD reactions were carried out in the ATC401 Thermal Cycler in 20 µl reaction volume containing 20 ng of genomic DNA as templates, 20 mM $MgSO_4$, 0.25 µM of primers, 200 µM of each dNTPs, and 0.5 unit of *Taq* polymerase (A&A Biotechnology, Poland). The RAPD procedure consisted of an initial denaturation at 91°C for 1 min, followed by 40 cycles for 15 s at 91°C, 15 s at 42°C, and 1 min and 10 s at 72°C. A final extension for 5 min at 72°C was performed. All reactions were carried out 2 times. After amplification, the reaction products were separated electrophoretically in 1.8% agarose gels stained with ethidium bromide in 1× TBE buffer, running at 100 V constant voltage for 3 h. A GeneRuler DNA Ladder Mix (100-10000 bp, Thermo Fisher Scientific) was used as a molecular standard. The size of the received products was assessed with the use

Table 1. RAPD primers used in molecular characterization of 28 *Capsicum* genotypes

	5′-3′sequences	Total number of bands	Number of polymorphic bands	Percentage of polymorphism	PIC
A01	CAGGCCCTTC	5	–	–	–
A02	TGCCGAGCTG	12	–	–	–
A03	AGTCAGCCAC	3	–	–	–
A04	AATCGGGCTG	10	4	40	0.436
A05	AGGGGTCTTG	9	5	56	0.357
A06	GGTCCCTGAC	4	–	–	–
A07	GAAACGGGTG	9	3	33	0.328
A08	GTGACGTAGG	11	2	18	0.278
A09	GGGTAACGCC	5	1	20	0.138
A10	GTGATCGCAG	6	1	17	0.268
A11	CAATCGCCGT	6	2	33	0.450
A12	TCGGCGATAG	7	3	43	0.282
A13	CAGCACCCAC	9	2	22	0.101
A14	TCTGTGCTGG	6	1	17	0.424
A15	TTCCGAACCC	5	2	40	0.374
A16	AGCCAGCGAA	8	2	25	0.192
A17	GACCGCTTGT	11	2	18	0.301
A18	AGGTGACCGT	12	–	–	–
A19	CAAACGTCGG	5	2	40	0.343
A20	GTTGCGATCC	9	–	–	–
AB9	GGGCGACTAC	10	2	20	0.307
AE11	AAGACCGGGA	7	3	43	0.386
B4	GGACTGGAGT	18	–	–	–
B10	CTGCTGGGAC	9	4	44	0.275
C15	GACGGATCAG	22	–	–	–
D12	CACCGTATCC	25	–	–	–
F5	CCGAATTCCC	8	3	38	0.109
K10	GTGCAACGTG	12	–	–	–
RAD1	TCCTACGCAC	6	2	33	0.499
RAD7	CAAACGTCCA	5	1	20	0.349
Total		274	47	–	–

of GelAnalyzer program version 2010a (gelanalyzer.com). The results were visualized and photographed using a Gel Doc 2000 UV transilluminator. Only bands that were bright and reproducible in both reactions were included in further analyzes (Fig. 1). The number of monomorphic and polymorphic amplification products generated by each primer was determined. The Polymorphic Information Content (PIC) was calculated according to a formula from Ghislain and coworkers (1999): $PIC = 1 - p^2 - q^2$, where p is the band frequency and q is no band frequency. Among all accessions, genetic distance coefficients were calculated according to the protocol by Nei and Li (1979). A dendrogram (Fig. 2) was constructed on the basis of the similarity matrix by the

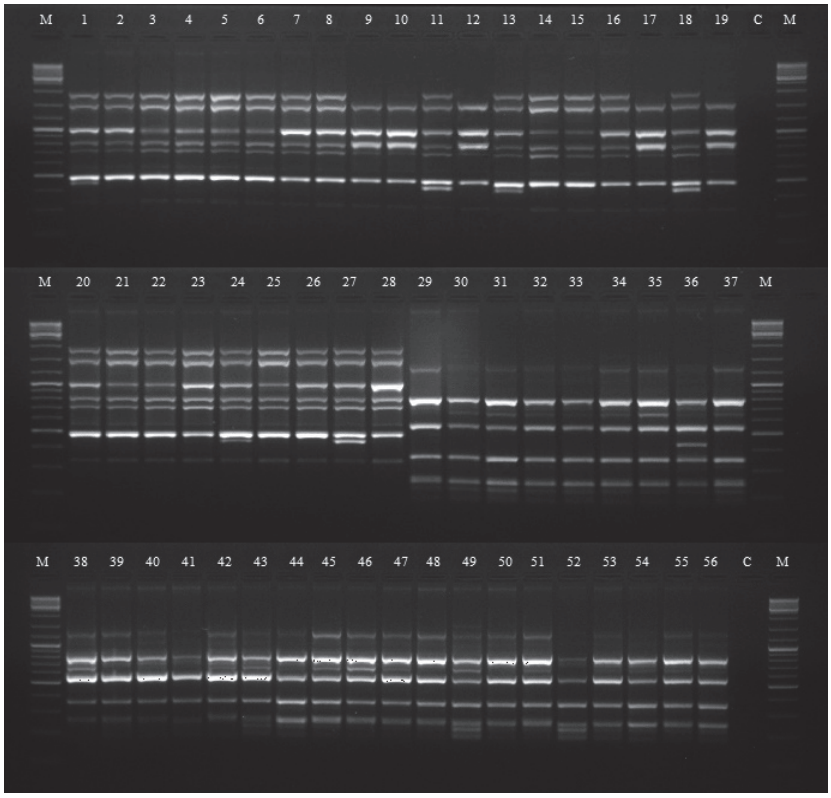

Fig. 1. RAPD bands obtained for primers A16 (lnes: 1-28) and A05 (lines: 29-56). DNA ladder (M); negative control (C); genotype: 3D (lines: 1, 38); 4D (2, 39); 5D (3, 40); 5H (4, 41); 6H (5, 42); 2D (6, 43); 7D (7, 44); 8H (8, 45); 20D 9, 46); 9D (10, 47); 10D (11, 48); 11D (12, 49); 12D (13, 50); 24D (14, 51); 17H (15, 52); 13H (16, 53); 16D (17, 54); 21D (18, 55); 25 D (19, 56); 22D (20, 29); 14D (21, 30); 23D (22, 31); 18H (23, 32); 1H (24, 33); 19D (25, 34); *C. annuum* L. ATZ1 (26, 35); *C. frutescens* L. (27, 36); (*C. annuum* L. ATZ1 × *C. frutescens* L.) F_1 (28, 37)

unweighted pair group method with the arithmetic average cluster analysis (Unweighted Pair Group Method with Arithmetic Mean, UPGMA) using the TREECON software package (Van de Peer and De Wachter, 1994).

Results

In the presented research, PCR reactions were carried out with 30 RAPD primers (Table 1). An example of the electrophoretic analysis of the amplified DNA using A16 and A05 primers is shown in Figure 1. All tested primers amplified the DNA from pepper accessions successfully. Depending on the primer, products of amplification were obtained from 3 (A-03) to 25 (D12). The size of the amplification products varied from 138 bp to 2369 bp. Twenty of 30 tested primers turned out to be appropriate for testing the diversity between different annual pepper genotypes. Of 247 RAPD products, 47 constituted polymorphic DNA fragments. The percentage of polymorphism between primers ranged from 17% (primer A14) to 56% (primer A05). The PIC value, which describes the informativeness of the primers, varied from 0.101 (primer A13) to 0.450 (primer A11). The applied reactions allowed to identify all the tested genotypes. Moreover, the amplification products were used for the calculation of genetic distances between plants. Pairwise distance matrices (Table 2) were computed using the TREECON software. The greatest distance (0.159) appeared between *C. frutescens* L. and the regenerant 12D, whereas the smallest (0.030) was observed between 2 androgenic plants, namely, 6H and 2D.

A dendrogram (Fig. 2) has been generated on the basis of Nei and Li formula using unweighted pair group method with the arithmetic average (UPGMA) cluster analysis. The data collected for 20 primers allowed to divide all the tested genotypes into two sections on the dendrogram (Fig. 2). One consisted of all androgenic regenerants: 18 diploids, 7 haploids, and the mother form of *C. annuum* L. ATZ1. The donor F_1 (*C. annuum* L. ATZ1 × *C. frutescens* L.) plant and *C. frutescens* L. parental plant belonged to the other group.

Table 2. Genetic distance estimates among 28 *Capsicum* genotypes

	1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	0.000																											
2	0.055	0.000																										
3	0.073	0.065	0.000																									
4	0.068	0.075	0.085	0.000																								
5	0.054	0.084	0.072	0.058	0.000																							
6	0.062	0.076	0.079	0.074	0.030	0.000																						
7	0.076	0.090	0.101	0.088	0.089	0.082	0.000																					
8	0.067	0.097	0.076	0.087	0.042	0.042	0.079	0.000																				
9	0.089	0.096	0.076	0.109	0.087	0.072	0.086	0.062	0.000																			
10	0.074	0.103	0.076	0.094	0.065	0.057	0.109	0.046	0.084	0.000																		
11	0.082	0.111	0.091	0.094	0.049	0.049	0.117	0.054	0.084	0.038	0.000																	
12	0.070	0.107	0.064	0.105	0.061	0.053	0.089	0.034	0.065	0.065	0.049	0.000																
13	0.110	0.124	0.126	0.099	0.100	0.100	0.107	0.105	0.112	0.104	0.104	0.115	0.000															
14	0.080	0.094	0.105	0.060	0.086	0.086	0.076	0.091	0.098	0.098	0.114	0.109	0.048	0.000														
15	0.108	0.090	0.116	0.080	0.097	0.097	0.128	0.126	0.133	0.133	0.141	0.144	0.115	0.100	0.000													
16	0.116	0.122	0.109	0.097	0.098	0.091	0.113	0.103	0.110	0.095	0.095	0.114	0.085	0.086	0.113	0.000												
17	0.120	0.110	0.120	0.116	0.102	0.102	0.140	0.115	0.098	0.098	0.098	0.132	0.080	0.089	0.109	0.072	0.000											
18	0.081	0.095	0.098	0.085	0.064	0.064	0.101	0.084	0.114	0.061	0.068	0.087	0.073	0.074	0.116	0.079	0.060	0.000										
19	0.092	0.106	0.116	0.097	0.068	0.068	0.120	0.095	0.132	0.079	0.079	0.105	0.092	0.101	0.104	0.098	0.071	0.041	0.000									
20	0.097	0.103	0.120	0.085	0.079	0.079	0.116	0.107	0.136	0.083	0.091	0.125	0.088	0.097	0.101	0.102	0.105	0.068	0.049	0.000								
21	0.067	0.081	0.076	0.055	0.057	0.057	0.110	0.070	0.108	0.077	0.077	0.080	0.105	0.083	0.094	0.103	0.099	0.076	0.080	0.084	0.000							
22	0.092	0.114	0.109	0.073	0.075	0.083	0.120	0.103	0.125	0.064	0.079	0.105	0.076	0.078	0.097	0.105	0.094	0.064	0.075	0.064	0.072	0.000						
23	0.085	0.098	0.086	0.104	0.075	0.068	0.104	0.080	0.094	0.072	0.087	0.075	0.099	0.101	0.120	0.083	0.109	0.071	0.104	0.101	0.080	0.075	0.000					
24	0.099	0.098	0.093	0.088	0.082	0.090	0.149	0.102	0.101	0.086	0.079	0.112	0.114	0.115	0.096	0.097	0.078	0.093	0.089	0.093	0.087	0.081	0.081	0.000				
25	0.099	0.120	0.100	0.119	0.082	0.082	0.119	0.079	0.101	0.056	0.064	0.090	0.114	0.115	0.126	0.067	0.086	0.071	0.089	0.093	0.102	0.096	0.052	0.074	0.000			
26	0.096	0.132	0.119	0.115	0.064	0.079	0.131	0.083	0.120	0.060	0.060	0.101	0.118	0.127	0.123	0.094	0.097	0.075	0.071	0.075	0.098	0.071	0.078	0.077	0.041	0.000		
27	0.130	0.120	0.123	0.165	0.157	0.134	0.119	0.147	0.124	0.124	0.139	0.134	0.159	0.154	0.157	0.127	0.115	0.115	0.126	0.145	0.147	0.141	0.111	0.110	0.096	0.114	0.000	
28	0.126	0.147	0.127	0.154	0.109	0.131	0.131	0.129	0.105	0.120	0.113	0.124	0.133	0.151	0.154	0.131	0.119	0.119	0.115	0.119	0.152	0.115	0.108	0.100	0.092	0.067	0.092	0.000

* 1-28 name of genotypes: 1 – genotype 3D, 2 – 4D, 3 – 5D, 4 – 5H, 5 – 6H, 6 – 2D, 7 – 7D, 8 – 8H, 9 – 20D, 10 – 9D, 11 – 10D, 12 – 11D, 13 – 12D, 14 – 24D, 15 – 17H, 16 – 13H, 17 – 16D, 18 – 21D, 19 – 25D, 20 – 22D, 21 – 14D, 22 – 23D, 23 – 18H, 24 – 1H, 25 – 19D, 26 – *C. annuum* L. ATZ1, 27 – *C. frutescens* L., 28 – (*C. annuum* L. ATZ1 × *C. frutescens* L.) F_1

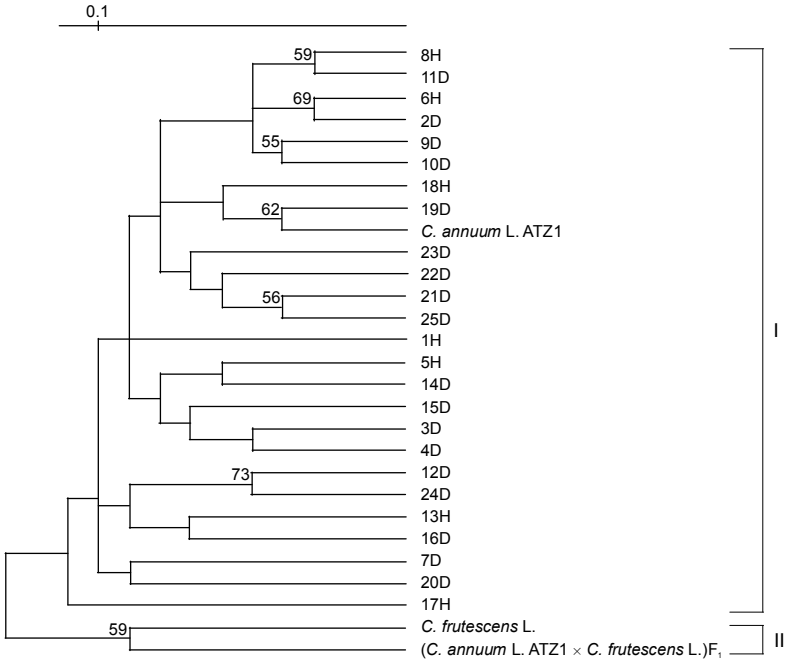

Fig. 2. Dendrogram showing genetic relationship among 28 *Capsicum* genotypes

Discussion

DH lines are the result of doubling the number of chromosomes of haploid plants, which results in their purely homozygotic character. This feature is especially precious in the process of breeding and seeds production. Conventional methods of obtaining such genotypes require self-pollination of the donor plants for many consecutive seasons, but they still do not guarantee the acquisition of fully homozygotic material, and significantly lower the plants vigor. The androgenic induction of embryos in anther cultures or isolated microspores allows to obtain fully homozygotic lines in one breeding season. The ability of embryogenic development of microspores in vitro depends, among others, on the plant species, the genotype, the method of maintaining cultures, and the composition of the medium. Among the obtained regenerants, there were haploid plants as well as some diploids and mixoploids (Olszewska et al. 2014). Spontaneous diploidization is a particularly favorable process as it eliminates the necessity of colchicine treatment and significantly reduces the time needed to obtain homozygotic lines. However, it requires an effective method of verification of the origin of the diploid regenerants. The variation of the morphological features or the analysis of isoenzymes (Olszewska et al. 2011, Odeigah et al. 1999) applied for this purpose are not always effective, especially in the case of genotypes with a small polymorphism level among these features. Molecular techniques detecting polymorphisms in the DNA are more precise. That is why they are successfully used to describe genetic variations between wild and cultivated pepper forms (Thul et al. 2009, Prasad et al. 2013), and to identify closely related *C. annuum* L. cultivars and hybrids (Sitthiwong et al. 2005).

The majority of contemporary breeded pepper varieties are F_1 hybrids. As pepper is a self-pollinating plant, the designing of hybrids requires an application of male sterile forms in the seed production process. Nevertheless, the instability of male sterility and its negative influence on the quality of the fruit do not allow for its widespread application in the case of pepper cultivars. Manual castration of flowers followed by crossbreeding is an arduous process and can easily lead to errors, which can result in genetically heterogeneous seeds. That explains the interest in finding molecular markers, which can be used as precise tools for controlling the genetic purity of hybrid seeds and identification of breeding cultivars. In the research carried out by Paran and coworkers (1998) and by Ballester and Vicente (1998), the utility of methods basing on a PCR reaction for the aforementioned purposes was assessed. Ilbi (2003) also proved the effectiveness of molecular techniques and

indicated the RAPD method as an efficient tool for assessing the purity of F_1 pepper seeds.

In the presented research, the PCR reaction was carried out with 30 primers. The products of 20 primers turned out to be appropriate to show the diversity between the tested genotypes, 47 products represented polymorphic DNA fragments. In the case of the identification of F_1 hybrid (*C. annuum* L. ATZ1 × *C. frutescens* L.), it was crucial to differentiate it from the maternal form to exclude the possibility of self-pollination. Therefore, the reaction products common for F_1 hybrid and *C. frutescens* L parental form were imparted our special attention. They were obtained as a result of a PCR reaction carried out with the following primers: A04, A07, A09, A10, A11, A17, and F05. Based on the received data, all tested genotypes have been divided into two sections on the dendrogram (Figure 2). The donor F_1 (*C. annuum* L. ATZ1 × *C. frutescens* L.) plant and *C. frutescens* L. parental plant belonged to the same group. The hybrid status of (*C. annuum* L. ATZ1 × *C. frutescens* L.) F_1 plant has been confirmed.

Defining identities of closely related genotypes is crucial in breeding programs. Lefebvre and coworkers (2001) investigated the genetic diversity between 47 genotypes of *C. annuum* L. using both the RAPD and the AFLP methods. The analysis of 136 RAPD primers resulted in 1204 PCR products and 544 (45%) of them were found to be polymorphic, 4 RAPD markers allowed to distinguish the analyzed pepper varieties. Lanteri and coworkers (2003) used 42 RAPD and 12 AFLP primers to identify 5 *C. annuum* L. populations. The effectiveness of both the methods was comparable. Eighteen RAPD primers allowed to obtain polymorphic products in the form of 159 bands, and 59 of them differentiated the tested genotypes. Therefore, it has been shown that the RAPD method can be successfully applied to identify genotypes within *C. annuum* L species.

In the presented experiment, the RAPD technique was applied to identify androgenic regenerants obtained in anther cultures of hybrid F_1 (*C. annuum* L. ATZ1 × *C. frutescens* L.). A cytometric analysis proved that of 25 regenerants 18 were diploid and 7 were haploid plants. In the case of diploid regenerants, it was crucial to exclude the possibility of their development from the somatic anther tissue and to confirm their microsporic origin. For haploid plants, genetic diversity was expected. These reactions provided all the necessary information on the regenerants obtained in the experiment. It was proved that all diploid regenerants differed from the anther donor plants. In the presented dendrogram (Fig. 2), they are all in one group with *C. annuum* L. ATZ1 genotype, which excluded their development from somatic anther tissues of the hybrid. At the same time, a genetic diversity between the obtained haploids was observed, which illustrated the genetic diversity of the microspores developing in hybrid anther cultures.

Conclusions

As a result of the preformed analyzes, the possibility of the development of diploid regenerants from somatic cells of the hybrid *C. annuum* L. ATZ1 × *C. frutescens* L. F_1 explants has been excluded, thus proving their microsporic origin and spontaneous diploidization. Moreover, their genetic diversity allowed to identify all the androgenic regenerants. Their closer relationship with the *C. annuum* L. ATZ1 mother plant of the hybrid was confirmed. On the basis of the results of the experiments carried out, it has been ascertained that the RAPD method is effective in identifying closely related pepper varieties.

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