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APPLICATION OF RAPD TECHNIQUE FOR IDENTIFICATION OF INTERSPECIFIC HYBRIDS FROM GENUS *Capsicum*

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Abstract. Molecular biology techniques based on DNA analysis are being increasingly used in modern plant breeding. In most cases DNA amplification methods using the PCR are being applied. The aim of this study was to assess the applicability of RAPD technique in identification of interspecific hybrids from genus *Capsicum*. The material consisted of selected interspecific hybrids (*C. frutescens* × *C. annuum*), (*C. frutescens* × *C. chinense*) and (*C. frutescens* × *C. baccatum*) and their respective parental breeding lines. The research was conducted with fifteen decamer primers. The size of amplified products ranged from 122–2127 bp, and their number from 3 to 17 per primer. All 15 primers made it possible to analyze 143 loci, of which 111 were polymorphic. The analysis of electrophoretograms allowed the identification of all studied genotypes and confirmation of hybrid origin of all tested hybrids. Two RAPD markers were obtained for the *C. frutescens* × *C. annuum* hybrid, two markers for *C. frutescens* × *C. baccatum* hybrid and five markers for *C. frutescens* × *C. chinense*. The data obtained were used to calculate the genetic distance between the investigated genotypes and construction of dendrograms.

Key words: molecular markers, PCR, hybrid seed testing, pepper, *Capsicum*

INTRODUCTION

The genus *Capsicum* is native to the American tropics, however, nowadays it is widely cultivated in tropical and temperate regions. It consists of 27 species, including 5 domesticated and 22 wild-type species [DeWitt and Bosland 1996]. After tomato, pepper is the world's second most important Solanaceae vegetable. Its large variability and great geographical distribution contributed to its multiple uses. Pepper can be consumed fresh, cooked or dried. It is used as an alimentary colorant or by the pharmaceutical industry. Recently, it is also being used as an ornamental plant. Annual global

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production of pepper is almost 30 million tons and it is cultivated on more than 1.8 million hectares [FAOSTAT].

Species identification in *Capsicum* genus is mainly based on selected morphological characteristics such as flower colour, seed colour, shape of the calyx and corolla, number of flowers per node and their orientation. Based on morphological characteristics, chromosome banding and hybridization studies *Capsicum* species have been classified into three complexes [Ince et al. 2009]. The *C. annuum* complex includes *C. annuum*, *C. frutescens* and *C. chinense*, which can hybridize with each other and are probably derived from a common ancestor. The *C. baccatum* complex includes the most common hot peppers (*C. baccatum* and *C. praetermissum*) of the Andean countries. The *C. pubescens* complex consists of highland species with purple flowers (*C. pubescens*, *C. eximium* and *C. cardenasii*) [Pickersgill 1997]. Species identification in taxa with a common ancestral gene pool is often difficult because of overlapping morphology and partial sexual compatibility [Pickersgill 1991]. Furthermore, morphological characteristics used as taxonomic descriptors are under the influence of environmental factors.

Interspecific hybridization is essential to the introduction of disease and pest resistance genes from wild and related species into commercial varieties, but also applied for increasing the nutritional quality, yield and adaptation to stress. It has been used to improve many Solanaceous crops despite the occurrence of some barriers to interspecific gene exchange such as unilateral incompatibility, post-fertilization embryo abortion and cytoplasmic male sterility in these genera [Pickersgill 1997]. Between *Capsicum* species interspecific crosses can be obtained with variable degree of success and crossability between species is not always reciprocal. Several interspecific hybrids were obtained within *C. annuum* complex, such as introducing TMV resistance from *C. frutescens* to *C. annuum* [Holmes 1937], resistance to PVY and TEV from *C. chinense* to *C. annuum* [Greenleaf 1956], partial resistance to CMV from *C. baccatum* to *C. annuum* [Caranta et al. 2002] or multiple-flower character from *C. chinense* into *C. annuum* [Tanksley and Iglesias-Olivas 1984]. Successful crosses between complexes are much more uncommon due to expression of unilateral or bilateral incompatibility [Djian-Caporalino et al. 2006].

Defining identity, purity and stability of varieties is an important part of hybrid seed production programme. Conventionally, genetic purity tests are conducted by field trials and based on morphological characteristics. This method is time consuming and space-demanding. Furthermore, morphological variation is often limited and does not allow the unambiguous identification of genotypes. Male sterility systems have been used for hybrid seed production in pepper, but the unstable behaviour of this trait and its negative effect on fruit quality did not allow avoidance of hand pollination. Manual emasculation and pollination procedures and hermaphrodite type of flowers, leave a chance for self pollination and fertilization.

Several molecular techniques have been used in pepper to distinguish hybrid plants from female parental lines. Isozymes and restriction fragment length polymorphisms (RFLPs) have proven to be useful tools for testing hybrid purity [Livneh et al. 1990]. Unfortunately, both techniques have some drawbacks: insufficient numbers of developed isozyme systems or low variability in some species and its environmental dependence. On the other hand, RFLPs have the limitations of high cost and complexity for

a large-scale commercial testing. PCR-based markers such as random amplified polymorphic DNA are relatively easy to perform and inexpensive. Furthermore, RAPD technique does not require the use of radioactive probes and can be applied without former knowledge of gene sequence. The genetic diversity among and within *Capsicum* species has been investigated in several studies using RAPD markers [Prince et al. 1995, Rodriguez et al. 1999, Ince et al. 2010]. Also the effectiveness of RAPD for routine assessment of variety identification and hybrid seed purity in closely related *C. annuum* cultivars has been tested previously [Ballester and de Vicente 1998, Ilbi 2003, Mongkolporn et al. 2004].

The main aims of this study are to: (1) assess the RAPD-based diversity within four tested *Capsicum* species, and (2) identify diagnostic RAPDs for taxonomic identification of accessions.

MATERIAL AND MATHODS

Plant material and genomic DNA isolation. Three interspecific hybrids: *C. frutescens* × *C. annuum*, *C. frutescens* × *C. baccatum*, *C. frutescens* × *C. chinense* and their correspondent parents were analyzed in this study. All plant material used in this survey were grown in a plastic tunnel at University of Technology and Life Sciences in Bydgoszcz under regular agronomical conditions. Two plants per accession were randomly sampled for DNA. Collected leaves were stored in –80°C for the isolation. Total genomic DNA was extracted from 100 mg of the youngest and healthy leaves using GenElute Plant Genomic DNA Miniprep Kit. The DNA solutions were stored at –20°C until analysis. Quality and purity of the extracted DNA was tested on 1.2% agarose gels stained with ethidium bromide.

PCR conditions. Total of 15 decamer primers were selected for this study. The PCR reactions were carried out with the ATC401 Thermal Cycler in 25 µl reaction volume containing 20 ng of genomic DNA as templates, 20 mM $MgSO_4$, 0.25 µM of primer, 200 µM of each dNTPs and 0.5 units of *Taq* polymerase (A&A Biotechnology, Poland). The RAPD profile consisted of an initial denaturation at 91°C for 1 min followed by 38 cycles consisting of 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 at least two times.

Data analysis. Amplified products were separated in 1.5% agarose gels in TBE buffer, running at 100 V constant voltage for 3 hours and stained with ethidium bromide. A MassRuler Express Reverse DNA Ladder Mix was included in each gel as a reference standard for band size. The results were visualized and photographed on a Gel Doc 2000 UV transilluminator. Only bands that were clear and reproducible, i.e., present (1) or absent (0) in both replications were included in further analysis. Genetic distance coefficients among all accessions were calculated according to Nei and Li [1979]. A dendrogram showing the patterns of genetic relationship between accessions was constructed on the basis of genetic distances matrix data by unweighted pair group method with arithmetic average (UPGMA) cluster analysis using the TREECON software package [Van de Peer and De Wachter 1994].

RESULTS AND DISCUSSION

A total of 15 primers were tested to identify polymorphism between hybrid varieties and its parental lines. All of tested primers successfully amplified the DNA from all accessions (figs 1–3). Depending on the primer 3 (A-03) to 17 (A-02) products of amplification were obtained. The A-03 primer was the only one to generate monomorphic band pattern. All 15 primers allowed for the analysis of 143 loci (tab. 1). The size of the amplification products varied from 122 to 2127 base pairs. The primers used for the analysis provided markers allowing for the distinction of all of the studied hybrids. Two markers were obtained for the *Capsicum frutescens* × *C. annuum* hybrid, two markers for *C. frutescens* × *C. baccatum* hybrid and five markers for *C. frutescens* × *C. chinense*. The highest number of distinct markers was obtained for the C. baccatum and *C. chinense* genotypes. The characteristic patterns obtained for these genotypes allow for performing fingerprinting with the use of the RAPD method in order to identify them. However, there has been little success in generating markers for distinction between the *C. annuum* and *C. frutescens*. All the markers, designated with primer number and the base pairs number of achieved bands, are listed in Table 2. We also obtained an electrophorogram for primer A-06 (fig. 1) which, on the basis of four polymorphic bands, allows for a swift and direct identification of the four parental genotypes used for the study (*C. annuum*, *C. frutescens*, *C. baccatum* and *C. chinense*).

The presence (1) or the absence (0) of the amplification products were used for the calculation of genetic distance between the genotypes. Pairwise distance matrices (tab. 3) were computed using TREECON 1.3b software. The program also generated a dendrogram (fig. 4) on the basis of Nei and Li formula using unweighted pair group method with arithmetic average (UPGMA) cluster analysis. The greatest genetic distance (0.500) was between *C. annuum* and *C. baccatum*, whereas the smallest (0.071) was observed between *C. annuum* and *C. frutescens* (tab. 3).

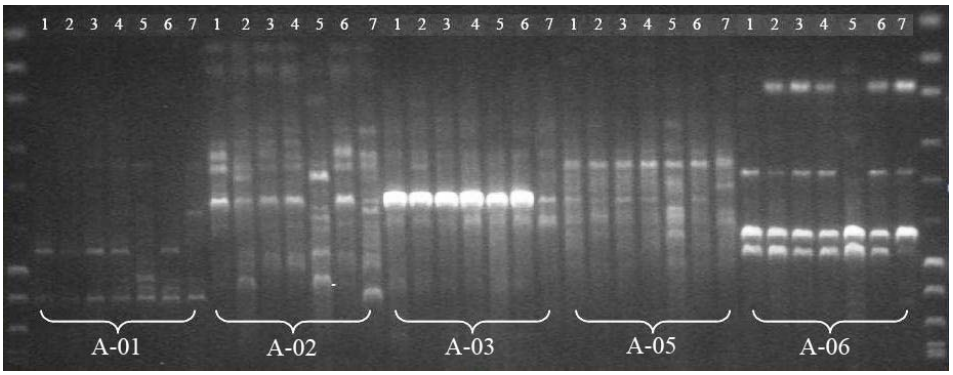

Fig. 1. RAPD patterns obtained for primers A-01, A-02, A-03, A-05 and A-06. From left to right: *C. annuum*, (*C. frutescens* × *C. annuum*) F1, *C. frutescens*, (*C. frutescens* × *C. baccatum*) F1, *C. baccatum*, (*C. frutescens* × *C. chinense*) F1, *C. chinense*. Molecular marker employed: 100, 200, 300, 500, 700, 1000, 1500, 2000, 3000, 5000, 7000, 1000 bp

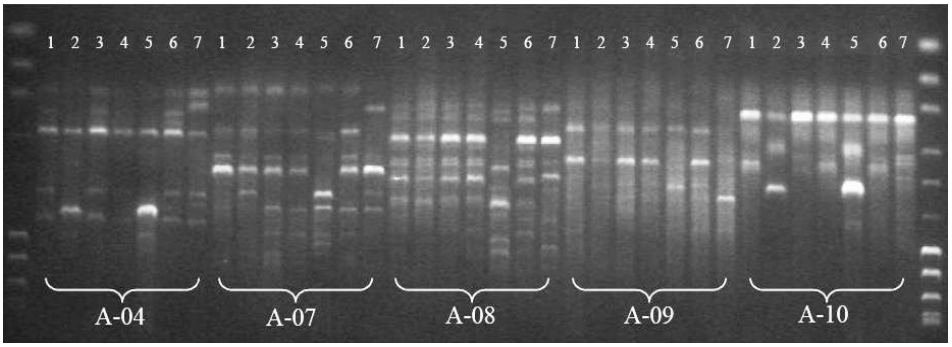

Fig. 2. RAPD patterns obtained for primers A-04, A-07, A-08, A-09 and A-10. From left to right: *C. annuum*, (*C. frutescens* × *C. annuum*) F1, *C. frutescens*, (*C. frutescens* × *C. baccatum*) F1, *C. baccatum*, (*C. frutescens* × *C. chinense*) F1, *C. chinense*. Molecular marker employed: 100, 200, 300, 500, 700, 1000, 1500, 2000, 3000, 5000, 7000, 1000 bp

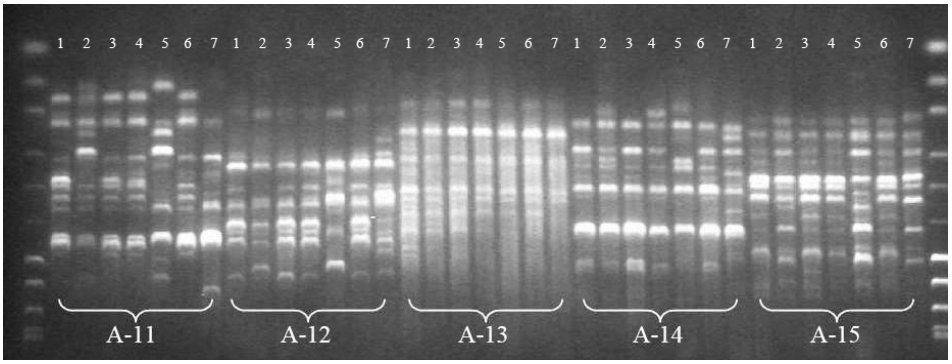

Fig. 3. RAPD patterns obtained for primers A-11, A-12, A-13, A-14 and A-15. From left to right: *C. annuum*, (*C. frutescens* × *C. annuum*) F1, *C. frutescens*, (*C. frutescens* × *C. baccatum*) F1, *C. baccatum*, (*C. frutescens* × *C. chinense*) F1, *C. chinense*. Molecular marker employed: 100, 200, 300, 500, 700, 1000, 1500, 2000, 3000, 5000, 7000, 1000 bp

The identification of analyzed materials is a significant issue in genetics, molecular biology and phylogenetics of plants. Molecular genetic markers are an important tool for solving this issue. Polymorphic DNA sequences are widely used, together with biochemical markers, for the analysis of plant genomes [Stachowiak and Świerczyński 2012, Rajesh et al. 2013]. The methods for generating molecular markers with the use of polymerase chain reaction and separation of amplified fragments on agarose or polyacrylamide gels are presently most common. Lately, the methods of multiple loci analysis, such as RAPD or AFLP, are most often used to determine the level of genetic polymorphism and phylogenetics [Kumar et al. 2009].

For a long time, the study of taxonomy and genetic distance of *Capsicum* species was based on easily distinguishable morphological characteristics such as shape and size of fruit. The use of morphology of fruit as taxonomical descriptor ceased when it

became clear that they do not correspond with other measures of genetic distance. Moreover, the looks of fruit are often influenced by selective pressure from the human factor which makes them unreliable evolutionary indicators [Stoffella et al. 1995]. Heiser and Smith [1953] designed the taxonomical system, that has remained in use ever since, which takes into consideration the morphology of the flowers, the ability to cross-breed and the fertility of the acquired distant hybrids.

Table 1. RAPD primers – sequence, melting temperature, total number of bands, number of polymorphic bands

Primer	5' → 3' sequences	T_m (°C)	Total number of bands	Number of polymorhic bands
A-01	CAGGCCCTTC	34	6	5
A-02	TGCCGAGCTG	34	17	16
A-03	AGTCAGCCAC	32	3	0
A-04	AATCGGGCTG	32	12	10
A-05	AGGGGTCTTG	32	6	3
A-06	GGTCCCTGAC	34	4	3
A-07	GAAACGGGTG	32	10	9
A-08	GTGACGTAGG	32	13	13
A-09	GGGTAACGCC	34	4	3
A-10	GTGATCGCAG	32	9	7
A-11	CAATCGCCGT	32	14	11
A-12	TCGGCGATAG	32	14	13
A-13	CAGCACCCAC	34	8	3
A-14	TCTGTGCTGG	32	12	9
A-15	TTCCGAACCC	32	11	6
Sum			143	111 (77.6%)

Table 2. RAPD markers designated with starter number and the number of base pairs

Species	Marker
C. baccatum	A-02$_{176}$, A-02$_{311}$, A-02$_{858}$, A-02$_{1014}$, A-02$_{1371}$, A-04$_{1782}$, A-05$_{406}$, A-07$_{978}$, A-08$_{337}$, A-08$_{642}$, A-08$_{1420}$, A-10$_{1109}$, A-11$_{219}$, A-11$_{1869}$, A-12$_{640}$
C. chinense	A-02$_{1619}$, A-04$_{328}$, A-04$_{687}$, A-04$_{984}$, A-10$_{550}$, A-10$_{686}$, A-11$_{2127}$, A-12$_{410}$, A-12$_{1344}$, A-14$_{379}$, A-14$_{825}$, A-14$_{1436}$
C. frutescens × *C. annuum* F1	A-12$_{485}$, A-13$_{606}$
C. frutescens × *C. baccatum* F1	A-01$_{1225}$, A-09$_{764}$
C. frutescens × *C. chinense* F1	A-01$_{767}$, A-02$_{422}$, A-08$_{1129}$, A-08$_{1308}$, A-09$_{880}$

The morphological analysis was used for the taxonomical identification and the determination of relation within the *Capsicum* genus [Eshbaugh 1993]. However, the qualitative characteristics are highly subjective and hard to score, while quantitative may be influenced by the environment. What is more, it has been observed that the place of cultivation has a direct influence on fruit, which causes difficulties in the morphological identification of different species of *Capsicum* [Zewdie and Bosland 2000, Todorova 2007]. Soller and Beckmann [1983] emphasise the possibility to use molecular markers as additional tools for describing varieties. Chromosome morphology [Pickersgill 1971], allozyme [Jensen et al. 1979] or isozyme markers [Loaiza-Figueroa et al. 1989] and electrophoretic analysis of seed proteins [Panda et al. 1986] are being used to assess genetic variation in genus *Capsicum*. Numerous reports describe the usefulness of molecular markers, such as restriction fragment length polymorphism (RFLP) [Lefebvre et al. 1993], amplified fragment length polymorphism (AFLP) or randomly amplified polymorphic DNA (RAPD) [Paran et al. 1998] for studying genetic variation in *Capsicum*. They are highly reliable in terms of measuring the genetic distance and may reflect the actual genetic differences better than morphological descriptors. In case of the *Capsicum* genus, the studies using the RAPD helped to identify genes being very important for the agronomics, the construction of genetic maps and the increase of knowledge about genetic diversity and phylogenetics of these taxa [Livingstone et al. 1999].

Table 3. Genetic distance values

Species	1	2	3	4	5	6	7
1 – *C. annuum*	x	0.215	0.071	0.120	0.500	0.095	0.321
2 – *C. frutescens* × *C. annuum*		x	0.207	0.210	0.333	0.207	0.361
3 – *C. frutescens*			x	0.046	0.447	0.080	0.299
4 – *C. frutescens* × *C. baccatum*				x	0.405	0.128	0.326
5 – *C. baccatum*					x	0.482	0.494
6 – *C. frutescens* × *C. chinense*						x	0.253
7 – *C. chinense*							x

Pattern interpretation of 15 primers products allowed for an unambiguous identification of the tested species. The amplified DNA of the *C. chinense* and *C. baccatum* showed its own specific pattern of bands, which allowed for distinguishing them from other species. The distinctness of RAPD profiles for these two species were associated with the appearance of additional fragments of DNA. For *C. baccatum* 15 such specific markers generated by eight primers, were obtained. In the case of *C. chinense* 13 unique markers generated by six primers were obtained. The specific markers for *C. annuum* and *C. frutescens* have not been observed. This situation may be caused due to small number of polymorphic loci observed for them or due to molecular technique limitation. Similar results were obtained by Rodriguez et al. [1999], who obtained diagnostic markers for all tested *Capsicum* species except for *C. frutescens*. Distinguishing be-

tween different species within one taxon originating from a common genetic pool (e.g. *C. annuum*, *C. frutescens* and *C. chinense*) may cause certain problems. According to Costa et al. [2006] RAPD markers amplify random DNA sequences which means that when two species or varieties are closely related, with a similar DNA sequence, minor differences in their genomes may not be detected in quantities allowing for distinction between them. Lanteri et al. [2003] suggest that the probability of detecting polymorphism depends on how alleles are associated to make up the diploid genotype. For a given number of alleles with known frequency of appearance the probability of detection is lowest in case of complete homozygotes. Due to the result of the reactions carried out only one allele may be detected as opposed to two alleles in heterozygote.

In order to confirm the purity of hybrid seeds it should be possible to distinguish, on the basis of a random sample, between seeds that have been obtained through cross-pollination of selected parental lines from those that were obtained as a result of self-pollination of female parent. Despite the dominant nature of RAPD markers, hybrid seed purity testing with the use of this technique is possible because only polymorphism associated with male parent should be taken into account. If the inbreed line was homozygous for the analyzed loci, all hybrids should also have a band associated with the loci. On the other hand, if the paternal line was heterozygous, half of the obtained hybrids can be considered mistakenly as the result of self-pollination of the female parent [Ballester and de Vicente 1998]. Jang et al. [2004] in order to obtain specific RAPD markers to assess the purity of hybrid seeds of *C. annuum* tested 200 primers that allowed them to select two primers generating markers common for the hybrid and the female parent and two other primers that generate bands specific to the hybrid form and the male parent. Ballester and de Vicente [1998], to assess the purity of hybrid seeds of five forms of *C. annuum*, used 100 primers. Only 53 gave clear amplification products, and among them 19 were polymorphic within the hybrids. From these polymorphic primers 8 were selected that generated amplification products common to the hybrid and the male parent. Also Mongkolporn et al. [2004] and Ilbi [2003], using a smaller number of primers (25 and 12 respectively) confirmed the possibility of using RAPD technique for identification of hybrids from an interspecific cross between *C. annuum* × *C. chinense* and intraspecific crosses between *C. chinense* and *C. annuum*. In our experiment, with the use of 15 primers, it was possible to obtain specific markers confirming hybrid origin of the studied genotypes. Two markers common to *C. annuum* and *C. frutescens* × *C. annuum* F1 hybrid, two markers specific for *C. frutescens* and *C. baccatum* × *C. baccatum* F1 and five markers for *C. chinense* and *C. frutescens* × *C. chinense* F1 were obtained. The results of molecular analysis, therefore, allowed for the determination of the nature of the hybrids, which could not be achieved by morphological analysis. However, due to a small number of acquired specific markers further studies should be carried out, with the use of greater number of primers, in order to confirm beyond doubt the origin of the analyzed hybrids.

The size and distribution of genetic variability within populations is a key aspect in understanding the origin and evolution of plant populations in natural conditions. Moreover, calculating genetic distance between existing cultivars is in the interest of their registration and protection, the protection of intellectual property, genetic banking as

well as broadening the genetic diversity of cultivars. The future of improving cultivars largely depends on accessibility and usability of the genetic variability [Lefebvre 2004].

The level of genetic diversity between cultivars depends, among others, on the method of pollination. Cross-pollinating crops show higher level of genetic diversity between cultivars. On the other hand, it is characteristic for self-pollinating crops to be of lower level of polymorphism [Lefebvre et al. 1993]. Despite the fact that pepper is a plant largely self-pollinated, genetic distance acquired through the analysis of RAPD profiles is considerable. The most phylogeneticly distant species of *C. annuum* and *C. baccatum* were 0.500 distant from each other. The lowest phylogenetical distance is between *C. annuum* and *C. frutescens* (0.071). The high values of genetic distance may be explained by rigorous pre-selection of RAPD primers and conditions for amplification.

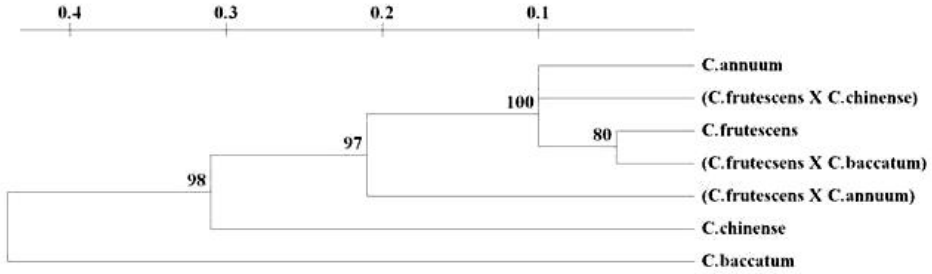

Fig. 4. Dendrogram generated by TREECON 1.3b software

Dendrograms acquired on the basis of the calculated genetic distance factors confirmed close relationship between *C. annuum, C. frutescens* and *C. chinense* species and their common classification under *C. annuum* complex, as well as putting *C. baccatum* in a different complex. Within the *C. annuum* complex, there was closer relationship between *C. annuum* and *C. frutescens* than *C. chinense*. Prince et al. [1995], who studying the polymorphism within the *Capsicum* genus, obtained similar results. On the basis of the dendrogram acquired through the RFLP and RAPD analysis he proved that *C. annuum* and *C. frutescens* are a part of the same group which is different and independent from the group containing *C. chinense* and *C. chacoesne*. Different results were obtained by Kochieva et al. [2004]. By analyzing dendrograms acquired through RAPD and AFLP analysis they came to a conclusion that there is closer relationship between *C. frutescens* and *C. chinense* than *C. annuum*. Therefore, the determination of phylogenetic relationships among the species of the *Capsicum* genus requires further study allowing for the unambiguous definition of the degrees of kinship.

Although pepper is a very important crop, the number of polymorphic loci detected so far is significantly below the average of many other crop species. Therefore, further studies in using different molecular techniques are needed in order to broaden our knowledge of the genome of this plant.

CONCLUSION

1. It has been shown that the RAPD technique allows for the identification of *C. frutescens* × *C. annuum*, *C. frutescens* × *C. baccatum* and *C. frutescens* × *C. chinense* interspecific hybrid forms as well as their parental lines.

2. Dendrograms and values of genetic distance generated through analyzing the results of RAPD reactions confirm close relationship between *C. annuum*, *C. frutescens* and *C. chinense* species.

3. It has been confirmed that there is a significant genetic diversity between the studied pepper species proved by the acquired values of genetic distance.

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WYKORZYSTANIE TECHNIKI RAPD W IDENTYFIKACJI MIESZAŃCÓW MIĘDZYGATUNKOWYCH W OBRĘBIE RODZAJU *Capsicum*

Streszczenie. We współczesnej hodowli roślin coraz częściej stosowane są techniki biologii molekularnej. W większości wypadków stosuje się metody wykorzystujące amplifikację DNA w łańcuchowej reakcji polimerazy. Celem niniejszej pracy była ocena przydatności markerów RAPD w identyfikacji mieszańców międzygatunkowych w obrębie rodzaju *Capsicum*. Materiał do badań stanowiły wybrane mieszańce międzygatunkowe (*C. frutescens* × *C. annuum*), (*C. frutescens* × *C. chinense*) (*C. frutescens* × *C. baccatum*) i odpowiednie linie rodzicielskie. Badania prowadzono z udziałem piętnastu 10-nukleotydowych primerów. Wielkość amplifikowanych produktów wahała się w granicach 122–2127 pz, a ich liczba od 3 do 17 w zależnościod primera. Wszystkie 15 primerów pozwoliło na analizę 143 loci, z których 111 było polimorficznych. Analiza elektroforogramów umożliwiła identyfikację wszystkich analizowanych genotypów oraz potwierdzenie mieszańcowego pochodzenia wszystkich badanych mieszańców. Otrzymano dwa markery RAPD dla mieszańca *C. frutescens* × *C. annuum*, dwa dla mieszańca *C. frutescens* × *C. baccatum* i pięć markerów dla mieszańca *C. frutescens* × *C. chinense*. Otrzymane dane posłużyły do obliczenia dystansu genetycznego między genotypami i konstrukcję dendrogramów.

Słowa kluczowe: markery molekularne, PCR, identyfikacja nasion mieszańcowych, papryka, *Capsicum*

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