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Estimation of genetic distance among genotypes of caraway (*Carum carvi* L.) using RAPD-PCR

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ABSTRACT. In order to estimate genetic diversity among starting materials and breeding strains of caraway, a collection of 17 accessions from botanical gardens in Europe, two cultivars 'Rekord' and 'Kończewicki', and four own breeding strains were analyzed by RAPD-PCR. The representative samples, each of five individual plants of accession, cultivar or strain, were taken from young rosette leaves. Forty of Genset Oligos RAPD primers were used for analysis and eight of them produced clear and reproducible banding patterns. In total, 62 banding patterns were obtained revealing 23 polymorphic bands, whereas the number of polymorphic bands ranged from two to four for one primer. The GS12 and GS43 primers generated two polymorphic bands, while each of the GS8, GS21, GS22, GS41 and GS53 primers generated three bands. The GS53 primer was the most informative one, revealing 60% of the estimated polymorphism. The estimated value of genetic distance ranged from 0.22 to 0.67. The lowest genetic distance was found between accessions from Cluj and Lousanne (0.22). The highest genetic distance was estimated between accession from Berlin and the strain no. 6 of cultivar 'Kończewicki' (0.67). UPGMA cluster analysis, based on eight RAPD primers, categorized the analyzed genotypes into four groups.

Keywords: *Carum carvi* L., genetic distance, genotype, RAPD.

Determinação da distância genética entre os genótipos de cominho (*Carum carvi* L.) por RAPD-PCR

RESUMO. Dezessete acessos oriundos de hortos botânicos na Europa, dois cultivares Rekord e Kończewicki, e quatro cepas foram analisados por RAPD-PCR para determinar a diversidade genética entre os materiais incipientes e as cepas do cominho. As amostras com cinco plantas individuais de acessos, cultivares ou cepas derivaram-se de jovens folhas em roseta. Quarenta Genset Oligos RAPD primers foram usados para análise, dos quais oito produziram padrões claros e reproduzíveis de banda. Sessenta e dois padrões de bandas foram obtidos, revelando 23 bandas polimórficas, enquanto o número de bandas polimórficas chegou entre dois a quatro para um primer. Os primers GS12 e GS43 prodiziram duas bandas polimórficas, enquanto cada um dos primers GS8, GS21, GS22, GS41 and GS53 gerou três bandas. O primer mais informativo foi GS53, revelando 60% do polimorfismo estimado. A distância genética estava entre 0,22 e 0,67. A menor distância genética encontrou-se entre os acessos oriundos de Cluj e Lousanne (0,22) e a maior distância genética foi estimada entre o acesso oriundo de Berlin e a cepa 6 do cultivar 'Kończewicki' (0,67). A análise de agrupamento UPGMA, baseada em oito RAPD primers, colocou os genótipos analisados em quatro grupos.

Palavras-chave: *Carum carvi* L., distância genética, genótipo, RAPD.

Introduction

Caraway (*Carum carvi* L.), a biennial herb of the family Apiaceae is widely cultivated in northern Europe, Russia, India, Canada and the United States and the world production of fruit may reach 15-20 thousand tones a year. In Poland caraway is the one of the most important cultivated medicinal plant which is cultivated on the area of 5000 ha and the fruit production is five thousand tones a year. The dried fruits of caraway (*Carvi Fructus*) are used mainly as a spice in food industry (SEIDLER-ŁOŻYKOWSKA; BOCIANOWSKI, 2012). In medicine caraway is used for its digestive, carminative, spasmolytic and stimulant properties. Limonene and carvone are the most important compounds of essential oil which is the main active substance of caraway fruit. Caraway is also the source of essential oil for cosmetic industry. Moreover, caraway oil is used as an alternative sprouting inhibitor during potatoes or flower bulb

storage (HARTMANS et al., 1995). Moreover, caraway could be a component of animal forage to enhance their well-being (SADOWSKA; OBIDOWSKA, 1998). Development of high yielding cultivars characterized by high content of active substances and better adapt to abiotic and biotic stress is the main aim of caraway breeding programs. The detailed evaluation of starting materials with respect to their genetic diversity is necessary for identifying the sources of variation. The assessment of genetic diversity with the use of molecular markers enables to define specific markers associated with, or linked to important agronomic and phenotypic traits which could be further used for selection.

Only a few species of medicinal plants were applied for analysis with respect to determination of genetic diversity using molecular markers, *i.e.,* marjoram (*Origamum majorana* L.), St. John's Wort (*Hypricum perforatum* L.), artichoke (*Cynara scolymus* L.), sweet basil (*Origanum basilicum* L.), chamomile (*Chamomila recutita* (L.) Rausch.) (ARNHOLDT-SCHMITT, 2002; KLÖCKE et al., 2002; MESSMER et al., 2002; WETZEL et al., 2002). Frequently, the selected molecular markers (random amplified polymorphic DNA, RAPD) are used for the detection of plant adulterants in some spice i.e. black pepper, chili or turmeric (DHANYA; SASIKUMER, 2010). Within the family of Apiaceae, genotypes of carrot (*Daucus carrota* L.) lines and accessions were characterized with the use of molecular markers. Bradeen et al. (2002) analyzed 124 carrot genotypes with 140 AFLP markers revealing high genetic diversity among wild type and population varieties. Grzebelus et al. (2001) used 33 RAPD and 88 AFLP markers for evaluation of genetic diversity among 31 carrot lines and accessions, as well as heterosis effect of three F_1 hybrids, in comparison with their parental lines. Based on the polymorphism level revealed by RAPD and AFLP analyzes, dendrograms showing genetic distance among the tested lines and accessions were constructed (GRZEBELUS et al., 2002; BARAŃSKI et al., 2004). As to our knowledge no results on molecular analysis of caraway genetic diversity were published yet. Although there were done some research in caraway doubled haploid (DH) lines by Ferrie et al. (2011) who investigated agronomic performance of 25 caraway DH lines in the field trials.

Here we present our results concerning assessment of genetic diversity among starting materials and breeding strains of caraway.

Material and methods

Plant material

A collection of 24 caraway accessions (Table 1) was established at the Institute of Medicinal Plants in Poznan.

Table 1. Origin of caraway collection (2007-2008).

Objects	Source
17 accessions	European botanical gardens of Bayreuth, Berlin, Bonn, Cluj, Cracow, Gottingen, Jena, Lousanne, Nantes, Poznan, Prague, Reykiavik, Riga, Salzburg, Ulm, Warsaw, Wroclaw
Cultivar 'Rekord'	Czech Republic
Cultivar 'Kończewicki' strain no. 6 and no. 7	The maintenance breeding of cv. 'Ko□czewicki' Institute of Medicinal Plants, Poland
Strains no. 9/10, no. 9/12, no. 9/13, no. 60/8	The breeding program Institute of Medicinal Plants, Poland

In 2007 and 2008 seeds of the collected accessions were sown in a greenhouse and 5-8-leaves plantlets were planted into a field at the beginning of May. In autumn, every year young rosette leaves of five plants from each accession were taken. Then these five leaves were mixed to make the bulk samples for DNA isolation.

RAPD-PCR

Amplification was performed in a reaction volume 25 µL containing 35 ng of primer, 1.5-4.5 ng DNA, 10 mM Tris-HCL, pH 8.3, 2 mM $MgCl_2$, 2.5 µg BSA, 100 µM of each dNTP, and 1.5 U of Taq DNA polymerase (Fermentas). PCR reaction, with the use of forty RAPD primers (Genset Oligos), was carried out in a Applied Biosystem thermocycler according to the following protocol: at 95°C for 5 min., 45 cycles: 95°C for 1 min., 35°C for 1 min., 72°C for 2 min., and final extension at 72°C for 5 min.

Amplification products were resolved by 1.5% agarose gel electrophoresis at 100 V by 2.5h. The 100-bp DNA Ladder Plus (GeneRuller^{TM}) was used as DNA fragment length determination.

Statistical analysis

The coefficients of genetic distance (D) for all caraway collection accessions were calculated according to the formula given by Nei (1972):

$$D_{N,AB} = \frac{2N_{AB}}{N_A + N_B},$$

where:
N_A is the number of bands present in accession *A*,
N_B is the number of bands present in accession *B*,
N_{AB} is the number of bands present in accessions *A* and *B*.

Based on calculated coefficients the accessions were grouped hierarchically using the unweighted pair group method of arithmetic means (UPGMA). The relationship among the accessions was presented as a dendrogram. The reliability of the similarity tree was assessed by the bootstrap method (FELSENSTEIN, 1985) with 1000 replications. We used 95% as the statistically significant values. Bootstrap analysis was performed by using the program MEGA 3.1.

Results and discussion

DNA of caraway collection accessions was tested by 40 RAPD primers and eight of them (GS8, GS12, GS21, GS22, GS38, GS41, GS43, GS53) revealed polymorphism (Table 2).

Table 2. Amplification products of RAPD primers (2007-2008).

Primer	Sequence 5' – 3'	Number of bands	Size of DNA (bp) min-max	Number and percentage of polymorphic bands
GS 8	AAAGCTGCGG	10	400-1500	3 (30.0)
GS 12	TCGGCGATAG	7	200-1300	2 (28.6)
GS 21	GGTGACGCAA	7	300-1500	3 (42.9)
GS 22	GTCTGACGGT	9	400-2000	3 (33.3)
GS 38	CAGGGGACGA	7	200-1500	4 (57.1)
GS 41	GTGGCTTGGA	8	200-1200	3 (37.5)
GS 43	GTGGCCGATG	9	200-1300	2 (22.2)
GS 53	ACGCCCAGAG	5	300-1500	3 (60.0)
total		62		23

In total, 62 of amplified DNA fragments were obtained, whereas 23 were polymorphic (37.1%). The number of polymorphic bands revealed by one primer ranged from two (GS12, GS43) to four (GS38). The size of amplification products ranged from 200 – 1500 base pair (bp). The GS53 primer revealed the highest polymorphism (60.0%), while the smallest one the GS43 (22.2%).

The genetic distance value estimated on the basis of RAPD markers ranged from 0.22 to 0.67 (Table 3). The lowest genetic distance was found between accessions from Cluj and Lousanne (0.22). Also low genetic distance values were found for accessions from Cluj and Bayreuth (0.26), Wroclaw and Jena (0.26) and Cluj and Prague (0.27). The highest genetic distance was revealed between accession from Berlin and strain no. 6 of cultivar 'Kończewicki' (0.67). High genetic distances were found between accession from Berlin and strain no. 9/13 (0.66) and between accession from Warsaw and strain no. 9/12 (0.66).

The dendrogram constructed on the basis of genetic distance sorted the tested accessions into four groups, with full statistical reliability – bootstrap value equal to 100% (Figure 1):

1. strain 9/12;
2. Cracow, cv. 'Kończewicki strain no. 6, strains 9/13, 60/8;
3. Riga, Bonn, strain 9/10;
4. Reykiavik, Berlin, Salzburg, Warsaw, cv. ‚Rekord', Jena, Wroclaw, Poznań, Gottingen, cv. 'Kończewicki strain no. 7, Nantes, Bayreuth, Cluj, Lousanne, Ulm, Prague.

Table 3. Genetic differentiation between 24 accessions of caraway collection (2007-2008).

Accession	Jena	9/2	Krakow	Poznan	Reykiawik	'Rekord'	Bareuth	Riga	Salzburg	Bonn	Ulm	Prague	Wroclaw	Gottingen	Warsaw	9/13	60/8	Kończewicki 6	Kończewicki 7	Berlin	Nantes	9/10	Cluj	Lousanne
Jena	0																							
Str. 9/12	0.56	0																						
Krakow	0.40	0.54	0																					
Poznan	0.32	0.49	0.31	0																				
Reykiawik	0.56	0.45	0.48	0.44	0																			
'Rekord'	0.46	0.47	0.47	0.37	0.52	0																		
Bayreuth	0.32	0.50	0.44	0.35	0.39	0.41	0																	
Riga	0.45	0.55	0.53	0.42	0.38	0.53	0.40	0																
Salzburg	0.37	0.53	0.49	0.42	0.48	0.46	0.41	0,46	0															
Bonn	0.36	0.57	0.49	0.42	0.57	0.56	0.37	0,32	0.39	0														
Ulm	0.39	0.46	0.37	0.34	0.35	0.43	0.43	0,45	0.40	0.45	0													
Prague	0.43	0.51	0.50	0.40	0.45	0.50	0.38	0,47	0.46	0.59	0.31	0												
Wroclaw	0.26	0.47	0.49	0.29	0.45	0.58	0.35	0,46	0.37	0.36	0.32	0.46	0											
Gottingen	0.35	0.50	0.46	0.30	0.48	0.32	0.38	0,46	0.32	0.40	0.38	0.44	0.43	0										
Warsaw	0.41	0.66	0.35	0.41	0.49	0.42	0.42	0,61	0.32	0.50	0.38	0.45	0.53	0.33	0									
Str. 9/13	0.45	0.62	0.39	0.42	0.61	0.59	0.55	0,64	0.48	0.48	0.48	0.49	0.52	0.39	0.43	0								
Str. 60/8	0.41	0.56	0.42	0.47	0.47	0.45	0.42	0,62	0.52	0.41	0.56	0.58	0.54	0.42	0.40	0.40	0							
Kończewicki str. 6	0.48	0.64	0.37	0.36	0.51	0.49	0.40	0,53	0.58	0.48	0.42	0.46	0.48	0.43	0.44	0.45	0.54	0						
Kończewicki str. 7	0.48	0.37	0.43	0.42	0.42	0.37	0.35	0,56	0.46	0.48	0.34	0.37	0.40	0.38	0.44	0.48	0.51	0.42	0					
Berlin	0.34	0.59	0.57	0.55	0.53	0.57	0.50	0,47	0.38	0.43	0.46	0.44	0.50	0.44	0.45	0.64	0.59	0.67	0.50	0				
Nantes	0.39	0.49	0.48	0.31	0.38	0.45	0.45	0,45	0.42	0.54	0.36	0.33	0.45	0.37	0.49	0.51	0.56	0.38	0.33	0.40	0			
Str. 9/10	0.40	0.61	0.47	0.49	0,52	0.47	0.35	0,43	0.43	0.30	0.46	0.56	0.43	0.44	0.54	0.49	0.48	0.52	0.43	0.44	0.48	0		
Cluj	0.38	0.41	0.49	0.35	0.39	0.44	0.26	0,43	0.41	0.49	0.32	0.27	0.38	0.33	0.50	0.48	0.54	0.40	0.35	0.41	0.40	0.35	0	
Lousanne	0.36	0.38	0.44	0.33	0.43	0.39	0.29	0.47	0.39	0.44	0.36	0.33	0.44	0.34	0.45	0.52	0.43	0.44	0.41	0.42	0.32	0.36	0.22	0

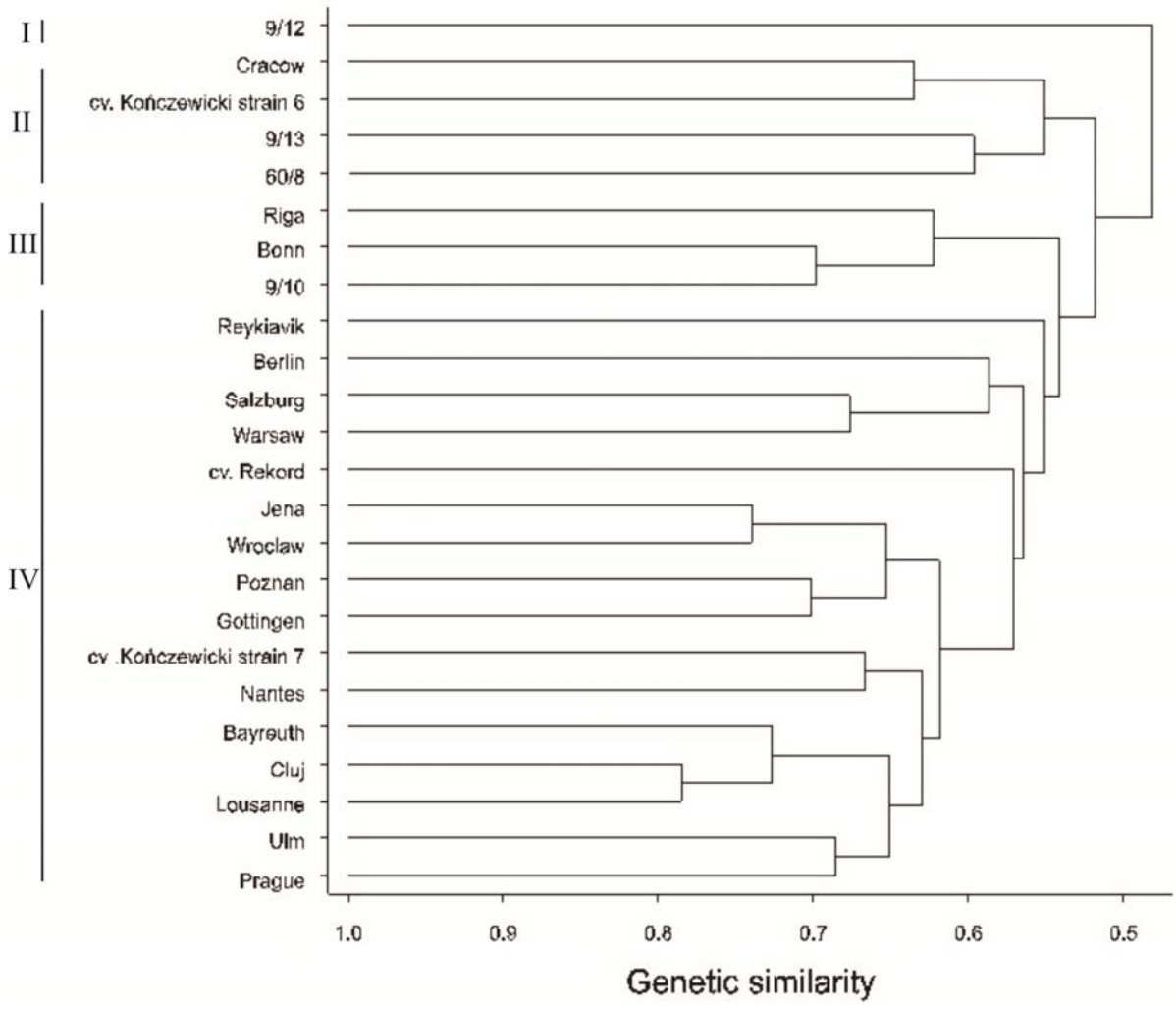

Figure 1. Cluster dendrogram of 24 caraway objects basing on eight RAPD primers (2007-2008).

RAPD can be applied for detection of genetic variation in many species and the estimated genetic distance value can be used for selection of starting materials in breeding programs (GRZEBELUS et al., 1997, 2001; BARAŃSKI et al., 2001; BOCIANOWSKI et al., 2003; NOWAKOWSKA et al., 2004; KUCZYŃSKA et al., 2007; BOCIANOWSKI et al., 2011; IRZYKOWSKA et al., 2012, 2013a and b; LIERSCH et al., 2013). In the presented research, a collection of 24 accessions, cultivars and breeding strains of caraway was analyzed using RAPD-PCR. The obtained results revealed significant differentiation of banding patterns among the collected caraway plants, thus enabling evaluation of genetic distance among them.

Up to now, no results have been published on caraway DNA analysis by RAPD-PCR, as well as by other molecular markers. Nevertheless, some medicinal plants species were analyzed by this method. Arnholdt-Schmitt (2002) determined genetic variability among St. John's wort (*Hypericum perforatum* L.) populations, using 44 RAPD primers. The 11 of St. John's wort populations in which determined 23 polymorphic bands were used for genetic distance evaluation (from 0.1054 to 1.0986). Moreover, RAPD markers linked to hypericine and flavonols content were identified. Sixteen RAPD primers were used for determining DNA polymorphism among marjoram (*Origanum majorana* L.) accessions (KLÖCKE et al., 2002) and the detected 128 polymorphic bands were applied for evaluation of genetic distance among the analyzed accessions. Twenty of Iranian black cumin (*Bunium persicum* Boiss.) accessions were analyzed with the use of 15 RAPD and 17 AFLP primers revealing 192 polymorphic bands by RAPD. The genetic distance ranged from 0.40 to 0.82 and it did not correlate with geographical distance of places of genotype origin (PEZHMANMEHR et al., 2009). Pirkhezri et al. (2010) reported the RAPD analysis of chamomile (*Chamomilla recutita* (L.) Rausch.) populations and cultivars determining 205

polymorphic bands by the selected 18 primers. The range of similarity varied from 0.15 to 0.78 and the populations were divided into two main groups. Lately, genetic variability of 32 cumin (*Nigella sativa* L.) genotypes was detected with the use of 58 RAPD primers, generating 164 (66%) polymorphic fragments. UPGMA cluster analysis indicated seven distinct groups (IQBAL et al., 2011). All the authors mentioned above pointed out that their studies provided important information for management of germplasm resources and could be used in breeding programs for crop improvement.

Conclusion

The presented in this work evaluation of genetic variation of the caraway collection will be useful for selection of appropriate plant materials in breeding programs and will be applied for future research on designing genetic markers linked to the important quality traits, as: carvone and limonene content.

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