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Preliminary data regarding genetic diversity of several endangered and endemic *Dianthus* species from Romania generated by RAPD markers

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SUMMARY. Conservation of endangered and endemic species of *Dianhtus* from Romania, requires the investigation of genetic polymorphism in the populations. Preliminary data were obtained by molecular characterization using RAPD markers. DNA amplification with the 9 RAPD primers of the individuals belonging to different populations of *D. callizonus, D. giganteus* ssp. *banaticus, D. glacialis* ssp. *gelidus, D. henteri, D. nardiformis, D. pratensis* ssp. *racovitzae, D. spiculifolius* and *D. tenuifolius* revealed low level of polymorphism within and between populations. Several polymorphic RAPD markers were identified being useful for investigation of genetic diversity. Out the 9 primers studied by us, only the primer OPB-07 ensured amplification in all species and primers OPA-13, OPE-04 and 1225 showed positive results in most of the species. The primers 4A-26 and 4A-27 ensured amplification only in *D. spiculifolius* and the primers 4A-23 and OPM-18 gave no results in none of the species.

Keywords: *Dianthus*, endemic/endangered species, genetic polymorphism, RAPD markers.

Introduction

Nowadays, in response to an alarming increase of species number with different degrees of endangerment, the conservation of biodiversity has a great

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importance. The concept of sustainable development implies the conservation of biodiversity both in situ and ex situ. Ex situ conservation is a viable alternative for highly endangered taxa in their natural environment, or may complete the in situ conservation methods (Marriott and Sarasan, 2010; Johnson *et al*., 2012; Butiuc-Keul, 2006; 2014; Cristea *et al*., 2014) that has consequences not only in science, but also in economy and culture. In order to develop a proper conservation management it is very important to know the genetic variability of in situ populations. In the case of in vitro conservation, it is also necessary to evaluate the somaclonal variability (i.e., the variability induced by the in vitro conditions) and to avoid the genetic uniformity. According to the International Union for Conservation of Nature (IUCN) there are approximately 33400 plant species threatened with extinction (Primack *et al*., 2008). Moreover, in 2014, there were 18291 plant species on IUCN red list (http://discover.iucnredlist.org/search?key=plants).

Genetic structure of plant populations, the level of genetic polymorphism within and among populations could offer valuable information in order to develop proper strategies for their conservation. The aim of conservation programs is to preserve a high number of populations, as well as their genetic structure and variability (Halmagyi and Butiuc-Keul, 2007; Butiuc-Keul, 2014). A characteristic of endangered and/or endemic plants is the maintenance of low levels of genetic variation in populations. Limited genetic diversity has been reported for many endangered and endemic plant species. The genetic structure and variability of endemic and rare plant populations were intensively studied by DNA markers (Luan *et al*., 2006; Breinholt *el al*., 2007; Cristea *et al*., 2014; Jarda *et al*., 2014).

Dianthus is one of the most diverse Mediterranean genera, more than 300 species were identified in Eurasia and Africa and over 100 species were found in Europe, more than 70 being endemic (Valente *et al*., 2010). In Romania, 58 *Dianthus* taxa have been recorded (Ciocârlan, 2009), of which 8 are endemic.

Our previous studies regarding the conservation of endemic and endangered species ensured valuable data for ex situ conservation strategies of several plant species of *Dianthus* as *D. giganteus* ssp. *banaticus* (Jarda *et al*., 2014), *D. henteri* (Cristea *et al*., 2010), *D. spiculifolius* (Butiuc-Keul *et al*., 2001; 2016; Cristea *et al*., 2006; 2009; 2013; 2014). Concerning the *Dianthus* genus our major interest was focused on the evaluation of genetic structure of populations and genetic variability within and between populations of several endemic and endangered species from Romania. Molecular markers as RAPD are valuable tools for such investigations because they are cost efficient, requires lower amounts of DNA and there is no necessary information about marker sequence. Thus, in this study we report the preliminary data regarding the usefulness of RAPD markers for the assessment of genetic polymorphism in the population of several *Dianthus* species from Romania.

Materials and methods

Plant material

The plant material was collected from different populations of the studied *Dianthus* taxa from Romania. Five individuals were collected from each location. Thus, *D. callizonus* was collected from 2 locations from Piatra Craiului Mountains: P1-Spîrlea refuge and Zăplaz (45°31'42.604"N, 25°12'00.174"E) and Diana refuge and Padina Popii, Piatra Craiului Mountains (45°33'29.184"N, 25°14'29.754"E); *D. giganteus* ssp. *banaticus* was collected from 3 populations: P1-Eşelniţa (44°39'27.99"N, 22°17'47.42"E), P2-Domogled Mt. (44º52'36.20"N, 22º26'13.00"E) and P3-Ţesna Gorge (44º58'03.30"N, 22º30'34.10"E); *D. glacialis* ssp. *gelidus* from 3 populations from Bucegi Mountains: P1-Omu Peak (45°22'51.004"N, 25°30'40.004"E), P2-Bâlea Lake, Făgăraş Mountains (45°31'40.004"N, 24°44'26.004"E), P3-Obârşia, Bucegi Mountains (45°22'50.004"N, 25°30'39.004"E); *D. henteri* from 3 populations from Vâlcea county: P1-Cornet (45°23'19.82"N, 24°18'28.54"E), P2-Călineşti Valey (45°22'19.34"N, 24°17'23.23"E), P3-Jiului Gorge (45°16'46.004"N, 23°23'19.004"E); *D. nardiformis* from 3 populations from Tulcea county: P1-Allah Bair (44°29'01.42"N, 28°13'24.88"E), P2-Consul Hill (45°10'55.19"N; 28°16'15.77"E), P3-Măcin (45°14'22.71"N, 28°35'01.01"E); *D. pratensis* ssp. *racovitzae* from 2 populations: P1-Dorobanţ village, Iassy County (47°14'33"N, 27°34'55"E), P2-Spătaru, Buzău County (45°4'14.56"N, 26°47'1.64"E); *D. spiculifolius* from 2 populations: P1-ROSCI0027 Natura 2000 site, Hăşmaş Mountains (46°44'274"N, 25°47'584"E), P2-ROSCI0002 Natura 2000 site, Apuseni Natural Park, Vlădeasa Massif (N 46°35'454"N, 22°48'384"E); *D. tenuifolius* from 2 populations from Suceava county: P1-Stânişoarei Mountains (47°21'50.87"N, 25°36'10.88"E), P2-Bistriţei Mountains (47°23'30.00"N, 25°30'16.65"E).

RAPD analysis

DNA was isolated using the CTAB method (Doyle and Doyle, 1987), RAPD markers were obtained by PCR amplification, performed in 25 µL of mixture containing 2 mM $MgCl_2$, 1 µM of each primer, 200 µM of each dNTP, 1.0 U of Taq (Fermentas) in reaction buffer (10mM TrisHCl pH 8.8, 50 mM KC1, 1.5 mM MgC12) and 25 ng of genomic DNA. Amplification programme: 1. T=94°C, 4 min; 2. T=94°C, 45 s; 3. primer alignment at 36 °C, 45 s; 4. elongation T=72°C, 50 s; steps 2-4, 35X. 9 primers have been tested as it can be seen in Table 1. Amplicons were separated on 1.5% agarose gel, stained with 0.5 µg mL^{-1} ethidium bromide. At least 2 independent PCR amplifications were performed for each primer.

Genetic similarities between individuals and populations were measured by Euclidian distance with the Past programme and the generated similarity coefficients were used for constructing a dendrogram with UPGMA option using the same programme.

Table 1.

Characteristics of primers used for RAPD amplification

No.	Primer sequence	Temperature of alignment
1	1225: 5'-AGGTGACCGT-3'	36 °C
2	OPA-13: 5'-CAGCACCCAC-3'	36 °C
3	OPB-07: 5'-GGTGACGCAG-3'	36 °C
4	OPE-04: 5'-GTGACATGCC-3'	36 °C
5	OPF-04: 5'-GGTGATCAGG-3'	36 °C
6	OPM-18: 5'-CACCATCCGT-3'	36 °C
7	4A-23: 5'-TCGCGAGCTG-3'	36 °C
8	4A-26: 5'-GTGATCGCAG-3'	36 °C
9	4A-27: 5'-CAATCGCCGT-3'	36 °C

Results and discussion

Amplification with the RAPD primers allows the identification of several polymorphic markers useful for investigation of genetic diversity between and within population. Out of the 9 primers studied by us, only the primer OPB-07 ensured amplification in all species. The specific patterns of amplification with this primer in *Dianthus* species are shown in Fig. 1.

The primer OPA-13 showed results in all species except *D. nardiformis*. The primers 1225 ensured amplification in most of the species except *D. henteri* and *D. pratensis* ssp. *racovitzae* and the primer OPE-04 also ensured amplification in most of the species except *D. giganteus* ssp. *gelidus* and *D. nardiformis*. Using the primer OPF-04 we obtained the successful amplification only in *D. callizonus*, *D. giganteus* ssp. *banaticus, D. giganteus* ssp. *gelidus, D. spiculifolius* and *D. tenuifolius*. The primers 4A-26 and 4A-27 showed amplification only in *D. spiculifolius* and the primers 4A-23 and OPM-18 gave no results in none of the species (Table 2). Regarding the polymorphism within and between populations, the results are different in the 8 studied *Dianthus* species.

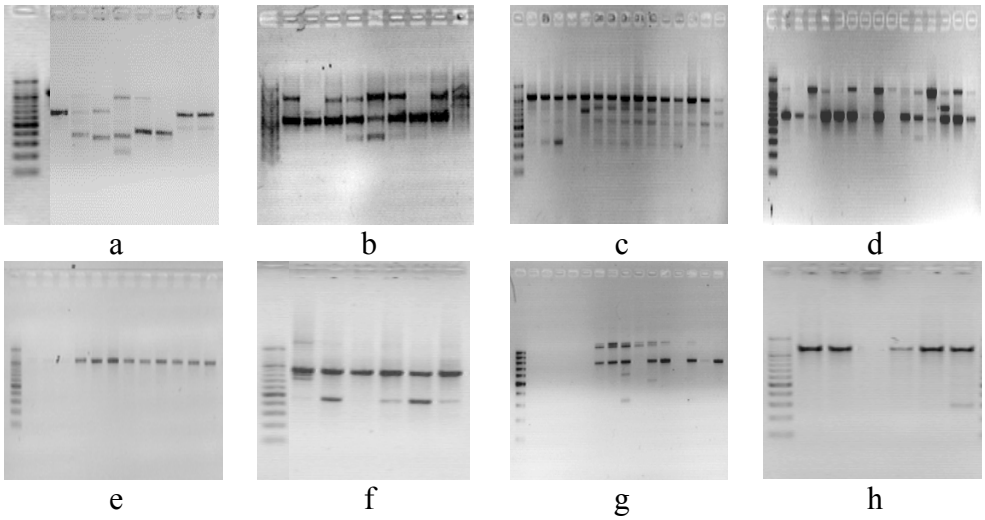

Figure 1. RAPD patterns of different *Dianthus* species with OPB-07 primer (a- *D.callizonus*: 1-molecular marker, 2-Dc 1.1, 3-Dc 1.2, 4-Dc 1.3, 5-Dc1.4, 6-Dc 1.5, 7-Dc 1.6, 8-Dc 1.7, 9-Dc 1.8; b-*Dianthus giganteus* ssp. *banaticus*: 1-molecular marker, 2-Dgb 1.1, 3-Dgb 1.2, 4-Dgb 1.3, 5-Dgb 2.1, 6-Dgb 2.2, 7-Dgb 2.3, 8-Dgb 3.1, 9-Dgb 3.2, 10-Dgb 3.3; c- *Dianthus giganteus* ssp. *gelidus*: 1-molecular marker, 2-Dgg 1.1, 3-Dgg 1.2, 4-Dgg 1.3, 5-Dgg 1.4, 6-Dgg 1.5, 7-Dgg 2.1, 8-Dgg 2.2, 9-Dgg 2.3, 10-Dgg 2.4, 11-Dgg 2.5, 12-Dgg 3.1, 13-Dgg 3.2, 14-Dgg 3.3, 15-Dgg 3.4, 16-Dgg 3.5; d- *Dianthus henteri*: 1-molecular marker, 2-Dh 1.1, 3-Dh 1.2, 4-Dh 1.3, 5-Dh 1.4, 6-Dh 1.5, 7-Dh 2.1, 8-Dh 2.2, 9-Dh 2.3, 10-Dh 2.4, 11-Dh 2.5, 12-Dh 3.1, 13-Dh 3.2, 14-Dh 3.3, 15-Dh 3.4, 16-Dh 3.5; e- *Dianthus nardiformis*: 1-molecular marker, 2-Dn 1.1, 3-Dn 1.2, 4-Dn 1.3, 5-Dn 1.4, 6-Dn 1.6, 7-Dn 2.1, 8-Dn 2.2, 9-Dn 2.3, 10-Dn 3.1, 11-Dn 3.2, 12-Dn 3.3; f-*Dianthus pratensis* ssp. *racovitzae*: 1-molecular marker, 2-Dpr 1.1, 3-Dpr 1.2, 4-Dpr 1.3, 5-Dpr 1.4, 6-Dpr 1.6, 7-Dpr 2.1; g-*Dianthus spiculifolius*: 1-molecular marker, 2-6-negative controls, 7-Ds 1.1, 8-Ds 1.2, 9-Ds 1.3, 10-Ds 1.4, 11-Ds 1.5, 12-Ds 2.1, 13-Ds 2.2, 14-Ds 2.3, 15-Ds 2.4, 16-Ds 2.5; h- *Dianthus tenuifolius*: 1-molecular marker, 2-Dt 1.1, 3-Dt 1.2, 4-Dt 1.3, 5-Dt 2.1, 6-Dt 2.2, 7-Dt 2.3; number significance: first number-population, second number-individual). Separation on 1.5% agarose gel stained with 0.5 μg/mL ethidium bromide.

Genetic variability in population of *D. callizonus* was assessed by RAPD markers generated with different primers as 1225, OPE-04, OPF-04, OPB-07 and OPA-13 (Table 2). All primers generated different polymorphic patterns but the highest polymorphism was observed with the primers OPE-04 and OPB-07. The dendrogram constructed based on Euclidian distance between individuals showed that they are clusterd in two groups of similarity (Fig. 2).

Table 2.

Genetic polymorphism of *Dianthus* species based on RAPD markers

Species	Population	RAPD fragments						
		1225	OPA-13	OPB-07	OPE-04	OPF-04	4A-26	4A-27
D.callizonus	P1	5	12	7	5	3	-	-
D. giganteus ssp. *banaticus*	P1	3	5	3	2	5	-	-
	P2	2	7	3	2	3	-	-
	P3	3	4	3	2	3	-	-
D. giganteus ssp. *gelidus*	P1	4	3	3	-	4	-	-
	P2	2	3	4	-	6	-	-
	P3	4	3	4	-	5	-	-
D. henteri	P1	-	3	2	5	-	-	-
	P2	-	4	2	4	-	-	-
	P3	-	4	4	4	-	-	-
D. nardiformis	P1	1	-	1	-	-	-	-
	P2	2	-	1	-	-	-	-
	P3	3	-	1	-	-	-	-
D. pratensis ssp. *racovitzae*	P1	-	1	4	3	-	-	-
	P2	-	1	4	1	-	-	-
D. spiculifolius	P1	5	6	5	5	4	2	4
	P2	2	4	2	5	3	2	3
D. tenuifolius	P1	2	3	1	2	2	-	-
	P2	2	3	2	3	5	-	-

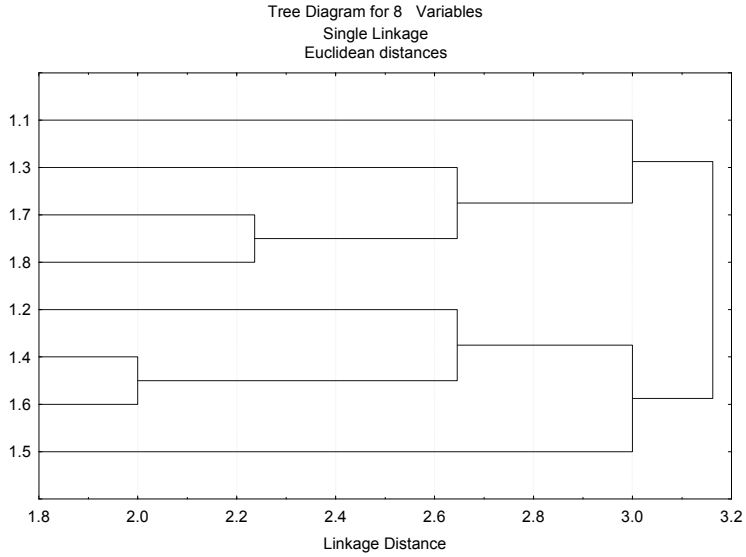

Figure 2. Dendrogram illustrating similarities between the individuals of *Dianthus callizonus* based on RAPD markers.

In *Dianthus giganteus* ssp. *banaticus*, RAPD amplification was successfully obtained only with the primers 1225, OPE-04, OPF-04, OPB-07 and OPA-13 (Table 2). The primer 1225 showed 3 bands in the populations P1 and P3, and only 2 bands in the population P2. There is one specific band which is present only in population P3-Cheile Ţesnei, Mehedinţi. The populations from Eşelniţa and Domogled are extremely similar, the individuals showing the same RAPD pattern with this primer. Similar RAPD patterns were also obtained in all populations with the primer OPE-04. The amplification with the primer OPF-04 showed differences between individuals and populations, thus this primers is appropiate for genetic polymorphism assessment. RAPD markers generated with the primer OPB-07 are present in all individuals from P2 population and in some individuals from P1 and P3 populations, these patterns being low polymorphic. High genetic polymorphism was showed by OPA-13 primer, while higher polymorphism being identified in the populations P1 and P2 than in the population P3. Based on RAPD markers a dendrogram of similarity between individuals was generated (Fig. 3). Thus, the individuals of *Dianthus giganteus* ssp. *banaticus* are clustering in 3 groups. All groups are including individuals from all populations. The populations P1 and P3 are more similar than the population P2. In conclusion the primers OPF-04 and OPA-13 showed high genetic polymorphism between and within populations. The primer OPB-07 showed only genetic polymorphism within populations.

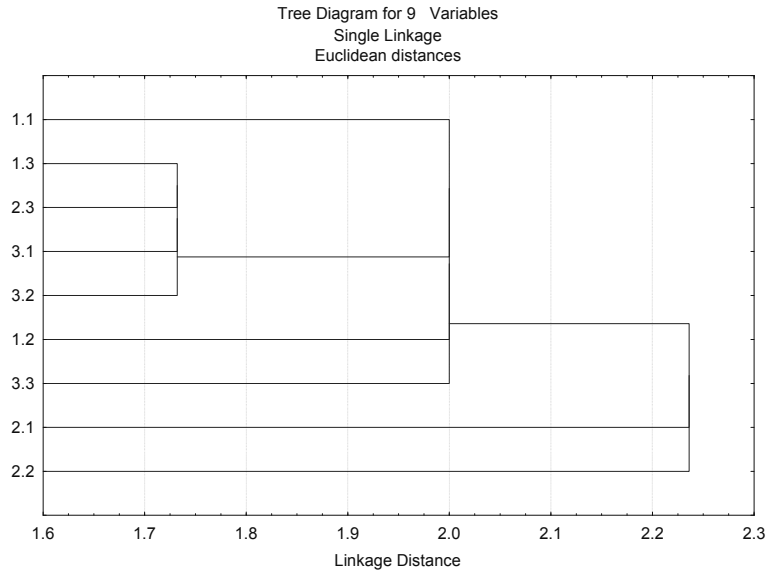

Figure 3. Dendrogram illustrating similarities between the individuals of *Dianthus giganteus* ssp. *banaticus* based on RAPD markers.

RAPD amplification of *Dinathus giganteus* ssp *gelidus* was successfully obtained with primers 1225, OPF-04, OPB-07 and OPA-13 (Table 2). Amplification with 1225 primer generated 7 different bands showing high level of genetic polymorphism between and within populations. The OPF-04 primer generated high number of bands with polymorphic distribution between populations. The OPB-07 primer generated 5 different bands that showed genetic polymorphism between populations. The primer OPA-13 showed the same pattern of the individuals from P3 population and low genetic polymorphism between individuals from P1 and P2 populations. The dendrogram of similarity showed that the individuals from P3 population and several individuals from P1 popultion are clustered together, while the individuals from P2 population are clustered together in a distinct group. Several individuals from P1 population are clustered in other group at higher genetic distance (Fig. 4).

RAPD amplification in *D. henteri* was successfully obtained only with the following primers OPE-04, OPB-07 and OPA-13 (Table 2). DNA aplification with the primer OPE-04 generated 5 bands that are present in several individuals, thus this primer showed low genetic polymorphism between and within populations. Similar results were also been obtained with the primer OPB-07, 4 different bands being obtained that are present in some of the individuals. High genetic polymorphism was observed by DNA amplification with the primer OPA-13, 4 fragments were observed but their distribution is very different in the individuals belonging to this plant species. Similarity dendrogram constructed based on RAPD markers showed that the individuals from all populations are very similar. The individuals are clustered in 3 groups, the first and the second contain most of the individuals, while the third group contains only few individuals (Fig. 5).

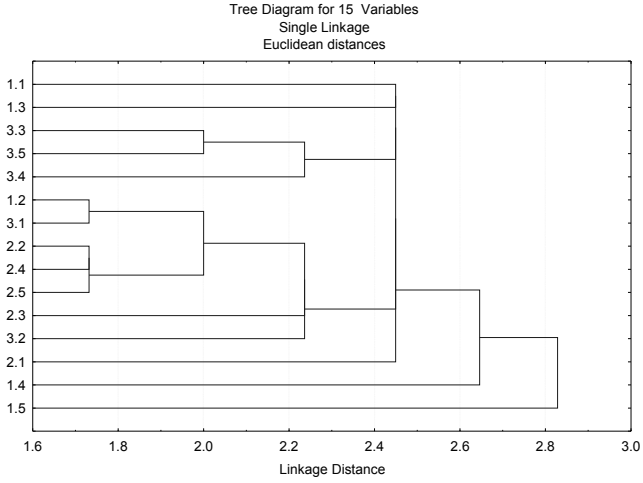

Figure 4. Dendrogram illustrating similarities between the individuals of *Dianthus giganteus* ssp. *gelidus* based on RAPD markers.

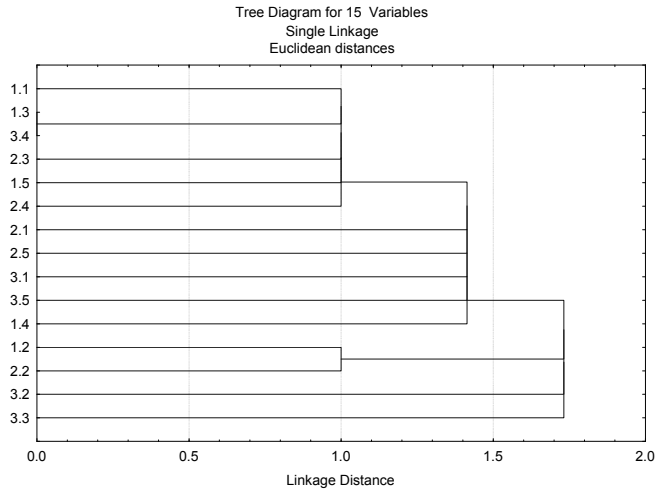

Figure 5. Dendrogram illustrating similarities between the individuals of *Dianthus henteri* based on RAPD markers.

Regarding the genetic variability in *D. nardiformis*, amplification with RAPD markers was very difficult to achieve, only 2 primers as 1225 şi OPB-07 allowed amplification with scorable bands (Table 2). By amplification with the primer 1225, only 2 bands were obtained, the first is present only in the individuals from P2 and P3 populations and the second one is present in all individuals independent on the population. Thus, this primer detected low genetic polymorphism between populations, but not within population. By amplification with OPB-07 primers, the RAPD patterns of all individuals are the same, except several individuals from the population P1. The genetic polymorphism detected only with the primer 1225, did not ensure the different clustering of individuals.

In *D. pratensis* ssp. *racovitzae* RAPD amplification was also very difficult, successfully amplification was achieved only with 3 primers as OPE-04, OPB-07 and OPA-13 (Table 2). The amplification with OPE-04 primer generated 3 different fragments, each of them identified in one individual. Amplification with the primer OPB-07 generated 5 fragments that show several differences between individuals. The primer OPA-13 generated only 1 fragment that is present in all individuals. Despite of other species this primer showed no polymorphism in this species. Unfortunately the number of individuals collected form each population is very small and no pertinent conclusion could be showed by these results. The individuals of this species are clustered in 2 groups, in wich are mostly the individuals from population P1. The individual from population P2 belongs to the first cluster (Fig. 6).

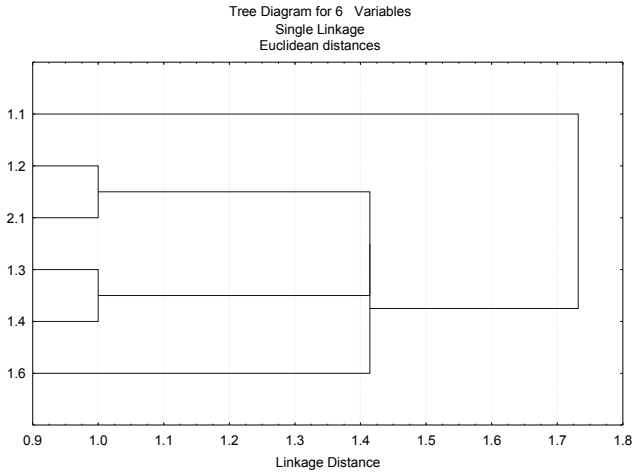

Figure 6. Dendrogram illustrating similarities between the individuals of *Dianthus pratensis* ssp. *racovitzae* based on RAPD markers.

In *D. spiculifolius* the RAPD amplification was achieved with the most of the primers, as 1225, OPF-04, OPE-04, OPB-07, OPA-13, 4A26 and 4A27 (Table 2). By amplification with 1225 primer we obtained 5 fragments that showed high genetic polymorphism of the individuals from both populations. Similar results were obtained with the primer OPF-04, 5 polymorphic fragments being identified. With the primer OPE-04, also 5 fragments were obtained, this primer showed genetic polymorphism within populations. By amplification with the primers 4A26 and 4A27 low genetic polymorphis was detected, thus the primer 4A26 generated 2 fragments, and the primer 4A27 generated 3 fragments. These primers showed genetic polymorphism between populations. The primer OPB-07 generated 6 fragment that showed genetic polymorphism between and within populations. The highest polymorphism was detected with the primer OPA13, 7 fragments being identified that showed genetic polymorphism within populations. Dendrogram of similarity shows that most of the individuals are clustered togheter in one group, and other group is formed only by the individuals 1.1, 2.1 and 2.3 (Fig. 7).

Amplification with RAPD primers of *Dianthus tenuifolius* was successfully obtained with the primers 1225, OPE-04, OPF-04, OPB-07 and OPA-13 (Table 2). The primer 1225 generated 2 fragment, the first fragment is present in all individuals and the second is present only in the individuals from Rodnei Mt. By amplification with OPE-04 primer, 4 fragments were obtained having a polymorphic distribution in the individuals belonging to different populations. The genetic polymorphism of the population P2 is very low. The amplification with the primer OPF-04 generated 5 fragments. The amplification with the primer OPB-07 showed only 2 fragments, the first being present in all individuals and the last one only in the individual 2.3.

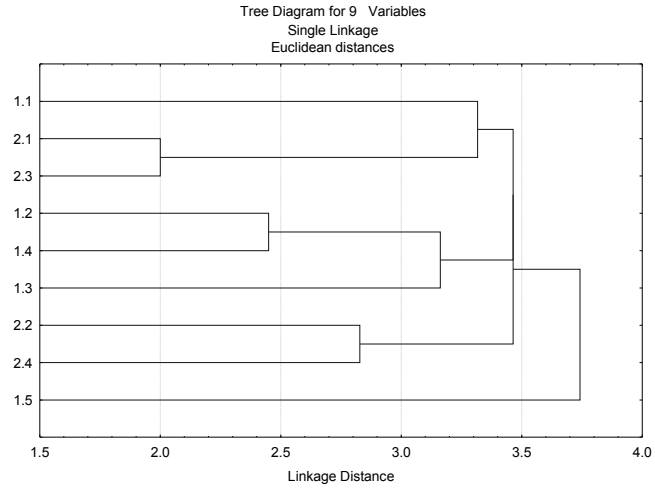

Figure 7. Dendrogram illustrating similarities between the individuals of *Dianthus spiculifolius* based on RAPD markers.

The primer OPA-13 generated 4 fragments with polymorphic distribution between individuals. The dendrogram of similarities showed 2 groups, one of them containing the individuals 1.3 and 2.1 and the secong group containing the individuals 1.1. and 2.2. The other individuals are more different from those included in these clusters (Fig. 8).

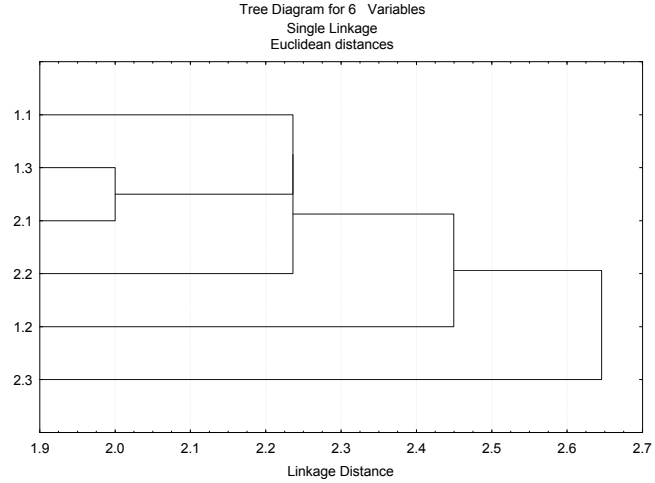

Figure 8. Dendrogram illustrating similarities between the individuals of *Dianthus tenuifolius* based on RAPD markers.

Despite of many morphological (Farsi *et al*. 2013) and cytological (Jafari and Behroozian, 2010) studies on different species of *Dianthus*, there are only limited information on the genetic diversity in the wild populations of these species. There are only few studies regarding the genetic diversity of *D. caryophyllus* using RAPD markers (Su Yeons, 2002) or *D. polylepis* and *D. crinitus* (Behroozian *et al*., 2013). Recent works revealed low genetic variability of 3 endemic and endangered species of *Dianthus* from Romania as D. *spiculifolius* Schur; D. *giganteus* d'Urv. subsp. *banaticus* (Heuff) and *D. callizonus* (Cristea *et al*. 2014; Jarda *et al*. 2014; Gabel *et al*. 2016).

Our preliminary data obtained with RAPD markers showed low level of polymorphism in the populations of the 8 *Dianthus* species. RAPD markers usually revealed high polymorphism even in the situation when other situs specific markers as SSR showed low polymorphism (Safari *et al*., 2013; Jarda *et al*., 2014) but was not the same in our study. Our results are in concordance with other data showing low diversity and polymorphism of *Dianthus* species from Iberian Peninsula (Balao *et al*., 2010) and Iran (Farsi *et al*., 2013) and to other data revealing low genetic polymorphism of plants having small populations isolated in fragmented mountain habitats (Duminil *et al*., 2007). Mountain and alpine plants have to cope with harsh environmental conditions and usually the low genetic diversity is compensated by clonal propagation, which produces rapid, but spatially limited spread of genotypes (Young *et al*., 2002; Gabel *et al*., 2016). This is also the situation of *Dianthus* species grown in severe environmental conditions (Stöcklin, 1992).

Conclusions

Genetic diversity of *Dianthus* species revealed by RAPD markers is generaly low. Out of 9 primers used in this study, only 6 generated reproducible and polymorphic patterns, useful for preliminary analysis of genetic polymorphism within and between populations.

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