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Genetic structure of populations of several endangered and endemic *Dianthus* species revealed by microsatellite markers

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Abstract – In order to develop a proper conservation programme for several endangered, rare or endemic species of *Dianhtus* from Romania, molecular characterization by simple sequence repeat (SSR) markers has been accomplished. Amplification of SSR loci in individuals belonging to different populations of *D. callizonus, D. glacialis* ssp. *gelidus, D. henteri, D. nardiformis* and *D. tenuifolius* revealed 23 polymorphic alleles. *D. callizonus* and *D. tenuifolius* showed particular sets of SSR alleles. *D. glacialis* ssp. *gelidus*, *D. henteri* and *D. nardiformis* proved to share almost the same alleles in most of the loci. The highest genetic diversity was observed in *D. glacialis* ssp. *gelidus* and *D. tenuifolius* in locus MS-DINMADSBOX. Allelic patterns across *Dianthus* species indicate that the mean number of different alleles was highest in *D. glacialis ssp. gelidus*, while the number of effective alleles was highest in *D. tenuifolius*. There are no particular differences in individuals belonging to the same species. Genetic diversity is generally low, ranging from 0.18 (*D. callizonus*) to 0.44 (*D. henteri*). Regarding the genetic diversity within populations of the same species, no differences were revealed by the use of the SSR markers tested in the present study.

Key words: *Dianthus*, endemic species, genetic structure, microsatellites, SSR markers, polymorphism

Introduction

In the last decades, habitat modifications induced by climate change and human activity led to the extinction of many plant species. Species conservation programs targeting large areas should focus on the most valuable populations and so require a better understanding of species biology and ecology as well as knowledge of the genetic diversity distribution within and among populations. Genetic diversity is important because it influences the populations' ability to adapt to a changing environment conditions. A characteristic of rare or endemic plants is the maintenance of low levels of genetic variation. Limited genetic diversity has been reported for many rare plant species (Hamrick et al. 1991, Frankham 1996, Leimu et al. 2006, Szczecińska et al. 2016). The genetic diversity within an endangered population is lost due to relatively faster genetic drift, which is exacerbated by limited gene flow. This can lead to inbreeding depression and higher homozygosity, which results in reduced adaptive potential (Reed and Frankham 2003, Johansson et al. 2007). Genetic diversity is often correlated with plant fitness (Leimu et al. 2006, Ilves et al. 2013).

Genetic structure of plant populations, the level of genetic polymorphism within and among populations could offer information of value in the development of proper strategies for their conservation. The aim of conservation programs is to preserve the highest number of populations, as well as their genetic structure and variability. The genetic structure and variability of endemic and rare plant populations were

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intensively studied by izoenzymes (Gitzendanner and Soltis 2000) and nuclear DNA markers (Pritchard et al. 2000, Cruzan 2001, Kang et al. 2005, °Cuan et al. 2006, Breinholt el al. 2007). Among these markers, simple sequence repeats (SSR) are frequently used to investigate genetic structure and variability in plant populations because of their cost effectiveness (Zietkiewicz et al. 1994, Varshney et al. 2005). There are many previous data regarding the usefulness of SSR in genetic studies in rare and endangered plants (Katoh et al. 2007, Sosa et al. 2010). Molecular characterization of the carnation was previously reported by sequence-related amplified polymorphism (SRAP) and inter simple sequence repeat (ISSR) markers (Fu et al. 2008). SSR markers have been also used for genotyping of carnation varieties (Smulders et al. 2003).

Dianthus is one of the most diverse Mediterranean-type plant genera; more than 300 species are distributed throughout Eurasia and Africa. Over 100 species of *Dianthus* species occur in Europe, and more than 70 are endemic (Valente et al. 2010). In Romania, 58 *Dianthus* taxa have been recorded (Ciocârlan 2009), of which 8 are endemic. Recent works have analysed the genetic variability of two endemic and endangered species of *Dianthus* from Romania (D. *spiculifolius* Schur; D. *giganteus* d'Urv. subsp. *banaticus* (Heuff) (Cristea et al. 2014, Jarda et al. 2014, Gabel et al. 2017).

In this paper we investigate the genetic diversity of 5 endemic and threatened Romanian *Dianthus* species using SSR markers. *D. callizonus* Schott & Kotschy, a Dacian element, is strictly endemic to the Curvature Carpathian Mountains. Previously classified as Vulnerable (VU) by Oprea (2005), it is currently considered as Low Risk (LR) species in the Red Book of Vascular Plants from Romania (Dihoru and Negrean 2009). *D. callizonus* grows on alpine and subalpine calcareous rocks. *D. glacialis* Haenke ssp. *gelidus* (Schott, Nyman & Kotschy) Tutin is a taxon endemic to the Eastern and Southern Carpathians (Oprea 2005). It is sporadic in alpine communities on calcareous rocks. *D. henteri* Heuff. ex Griseb. & Schenk is endemic to the Southern Carpathians. This species grows on rocky soils, in the beech and spruce belt (Sârbu et al. 2013). *D. nardiformis* Janka grows on rocky areas, in the Lower Danube Basin and in the Dobrudja region. This species is considered VU (Oprea 2005, Dihoru and Negrean 2009). *D. tenuifolius* Schur is an endemic species in the Romanian Carpathians and grows in meadows, on skeletal soil. It is considered a Least Concern (LC) species by Oprea (2005). All taxa are presumably diploids (2n=30), according to Carolin (1957), a ploidy feature that may allow an easy interpretation of their SSR patterns. All the above mentioned taxa are perennial and were identified in different Natura 2000 sites from Romania. Besides *D. nardiformis*, which is found not only in eastern Romania but also in northern Bulgaria, all the other 4 species are found only in Romania and are strictly endemic to the above mentioned areas.

Materials and methods

Plant material

The plant material was collected from different populations of the studied *Dianthus* taxa from Romania (Fig. 1).

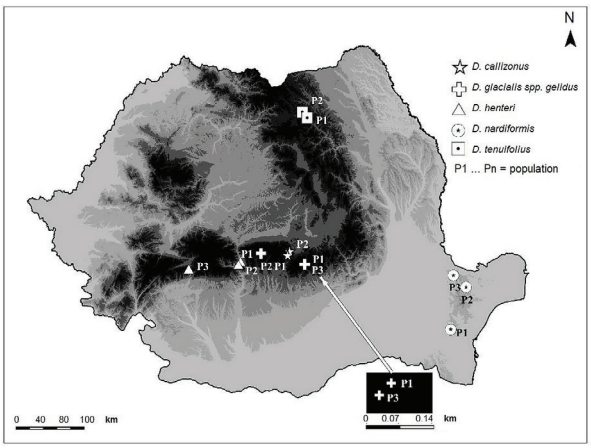

Fig. 1. Sampling locations for *Dianthus* populations in Romania.

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°31'42.60" N, 25°12'00.17" E) and Diana refuge and Padina Popii (45°33'29.18" N, 25°14'29.75" E); *D. glacialis* ssp. *gelidus* from 3 populations from Bucegi Mountains: P1-Omu Peak (45°22'51.00" N, 25°30'40.00" E), P2-Bâlea Lake, Făgăraş Mountains (45°31'40.00" N, 24°44'26.00" E), P3-Obârşia, Bucegi Mountains (45°22'50.00" N, 25°30'39.00" E); *D. henteri* from 3 populations from Vâlcea county: P1-Cornet (45°23'19.82" N, 24°18'28.54" E), P2-Călineşti Valley (45°22'19.34" N, 24°17'23.23" E), P3-Jiului Gorge (45°16'46.00" N, 23°23'19.00" 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. 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).

SSR analysis

Genomic DNA was isolated from leaves using the CTAB method described by Doyle and Doyle (1987). For SSR analysis, a total of five primer pairs (On-line Suppl. Tab. 1) were used (Smulders et al. 2000). PCR amplifications were performed in a 0.2 mL tube containing 2 mM $MgCl_2$, 1 μM of each primer, 200 μM of each dNTP, 1.5 U of Taq (Fermentas) and 25 ng of genomic DNA in a final volume of 25 μL. DNA amplification was performed according to the following program: 1. T = 94 °C, 5 min; 2. T = 94 °C, 45 s; 3. primer annealing at 55 °C, 45 s; 4. elongation T = 72 °C, 45 s; steps 2–4 were repeated 35 times. 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.

Data analyses

Several genetic diversity parameters were calculated for each locus and per *Dianthus* species using POWERMARKER 3.25 (Liu and Muse 2005). These included gene diversity (expected heterozygosity), observed heterozygosity (referred

Tab. 1. Genetic characterization of *Dianthus* species based on SSR alleles. Different types of alleles: *a*-BSY-150, *b*-BSY-100, *c*-BSY-75, *d*-BSY-50, *e*-DIA-200, *f*-DIA-175, *g*-DIA-100, *h*-DIA-75, *i*-DIA-50, *j*-DINCA-200, *k*-DINCA-150, *l*-DINCA-100, *m*-DINCA-50, *n*-DINGSTA-200, *o*-DINGSTA-175, *p*-DINGSTA-100, *r*-DINGSTA-75, *s*-BOX-300, *t*-BOX-175, *u*-BOX-150, *v*-BOX-100, *x*-BOX-75, *z*-BOX-50, - absence of allele.

Species/population		Location	No. of individuals	MS-DCAMCRBSY	MS-DCDIA30	MS-DINCARACC	MS-DINGSTA	MS-DINMADSBOX
D. callizonus	P1	Spîrlea refuge and Zăplaz, Diana refuge and Padina Popii, Piatra Craiului Mountains	5	1-2 alleles: 100:75 (*-b, bc)*	2 alleles: 100:75 (*gh*)	1 allele: 100 (*l*)	1 allele: 100 (*p*)	1 allele: 100 (*v*)
D. glacialis ssp. *gelidus*	P1	Omu Peak, Bucegi Mountains	5	0-1 alleles: 100 *(b-)*	1-2 alleles: 200:50 *(ei, -i)*	1-2 alleles: 200:50 *(jm, -m)*	1 allele: 75 *(r)*	0-2 alleles: 150:100:75 *(vx, ux, --, x-)*
	P2	Bâlea Lake, Făgăraş Mountains	5	1-2 alleles: 100:75 (*b-, bc)*	1-2 alleles: 200:50 *(ei, -i)*	1-2 alleles: 200:50 (*jm, -m)*	1 allele: 75 *(r)*	1-2 alleles: 150:75:50 (*uz, x-)*
	P3	Obârşia, Bucegi Mountains	5	1-2 alleles: 150:50 *(ad, b-)*	1-2 alleles: 200:50 (*-i, ei)*	1-2 alleles: 200:50 *(jm, -m)*	1 allele: 75 *(r)*	1-2 alleles: 150:75:50 *(uz, -z, x-)*
D. henteri	P1	Cornet, Vâlcea county	5	2 alleles: 100:50 (*bd)*	1-2 alleles: 200:75 (*eh, -h)*	2 alleles: 200:150:100 (*jk, kl)*	2 alleles: 200:75 (*nr)*	1-2 alleles: 150:100 *(uv, -v)*
	P2	Călineşti Valey, Vâlcea county	5	2 alleles: 100:50 (*bd)*	1-2 alleles: 200:75 (*eh, -h)*	2 alleles: 200:150:100 (*jk, kl)*	2 alleles: 200:75 (*nr)*	1-2 alleles: 150:100 *(uv, -v)*
	P3	Jiului Gorge	5	2 alleles: 100:50 (*bd)*	0-1 alleles: 75 *(-h)*	2 alleles: 150:100 *(kl)*	1-2 alleles: 200:75 *(nr, -r)*	0-1 alleles: 100 (*-v)*
D. nardiformis	P1	Allah Bair, Tulcea county	5	0-1 alleles: 100 (*-b, --)*	1-2 alleles: 200:75 *(-h, eh)*	1-2 alleles: 150:100 *(-l, kl)*	1-2 alleles: 200:100 *(n-, np)*	1-2 alleles: 150:75 (*-u, ux)*
	P2	Consul Hill, Tulcea county	5	0-1 alleles: 150:100 (*a-, -b)*	1-2 alleles: 200:75 *(-h, eh)*	1-2 alleles: 150:100 (*kl, k-)*	1-2 alleles: 200:100 (*n-, np)*	1-2 alleles: 150:75 (*-u, ux)*
	P3	Măcin, Tulcea county	5	0-1 alleles: 100 *(a-)*	1-2 alleles: 200:75 *(-h, eh)*	1-2 alleles: 150:100 *(-l, kl)*	1-2 alleles: 200:100 *(-p, np)*	1-2 alleles: 150:75 *(-x, ux)*
D. tenuifolius	P1	Stânişoarei Mountains, Suceava county	5	0-1 alleles: 100 *(a-)*	2 alleles: 175:75 *fh*	0-1 alleles: 100 *(l, --)*	1-2 alleles: 200:175:75 *(or, r-, no)*	1-2 alleles: 300:150:75 (*-x, ux, su)*
	P2	Bistriţei Mountains, Suceava county	5	1-2 alleles: 150:75 *(a-, ac, -c)*	0-1 alleles: 75 (*-h)*	0-1 alleles: 100 *(l, -)*	1-2 alleles: 175:75 *(or, -r)*	2 alleles: 300:150 *(su)*

to as heterozygosity), and polymorphism information content (PIC) value. These parameters take into account allele frequencies. Within species, richness and evenness were also explored using GENALEX6.5 (Peakall and Smouse 2006, 2012). The number of alleles per species (arithmetic mean across loci), the number of alleles with a frequency greater than 5%, the effective number of alleles, the number of private alleles and the number of locally common alleles occurring in less than 50% of the populations were explored. Genetic diversity was assessed via Shannon's information index, on a single-locus basis, based on natural logarithmic allele frequencies. The evolutionary history was inferred using the neighbour-joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 100.13433838 was constructed. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were provided by the user. Evolutionary analyses were conducted in MEGA 6 (Tamura et al. 2013). Principal coordinate analysis (PCoA) was used to explore multivariate

relationships among inter-individual genetic distances within each species and among *Dianthus* populations. Additionally, divergence statistics were computed using the analysis of molecular variance (AMOVA) implemented in ARLEQUIN 3.5 (Excoffier and Lischer 2010).

Results

Genetic polymorhism

All loci amplified well in nearly all individuals tested. A total of 23 polymorphic alleles were amplified. The number of alleles per locus and the frequency in each species is shown in On-line Suppl. Tab. 2. The average number of alleles was 4.6. The genetic variation within populations is generally low. Almost all the individuals belonging to a population share the same alleles. The allelic composition of each SSR locus based on the results of all five *Dianthus* species is detailed in Tab. 1. The most frequent allele in the locus BSY was *b* in all species except *D. tenuifolius* and the allele *d* had the lowest frequency, being present only in *D. henteri* individuals. In the locus DIA the most frequent allele was *h*, while the alleles *f* and *g* had the lowest frequencies. In the locus DINCA the most frequent allele was *l* and the least frequent was *m*. The most frequent allele in the locus DINGSTA was *r* and the lowest frequency was observed for the allele *o*. In the locus BOX the highest frequency was observed for allele *x* and the lowest frequency for allele *z*. The alleles *b, l* and *v* were present in most of the species, while the allele *g* was present only in *D. callizonus.* Individuals of *D. glacialis* ssp. *gelidus* showed a particular combination of alleles in most of the loci, the alleles *i* and *m*. Other alleles, *j* and *r,* were present only in some individuals of *D. henteri* and *D. tenuifolius.* The individuals belonging to *D. henteri* species showed the particular allele *d* and the allele *v* which was present in *D. callizonus* as well. *D. tenuifolius* showed a particular combination of alleles, the allele *a* which was also present only in *D. nardiformis* and the allele *c* also present in *D. callizonus*. The alleles *f, o* and *s*, were present only in *D. tenuifolius*.

In our study the gene diversity is low, ranging from 0.18 to 0.44, the lowest gene diversity being observed in *D. callizonus* (Tab. 2). The proportion of the heterozygous individuals (observed heterozygosity) ranged between 0.3 and 0.57, the lowest value being observed again in *D. callizonus.* The distribution power of the SSR marker, estimated by the polymorphism information content (PIC) value ranged between 0.14 (*D. callizonus*) and 0.34 (*D. henteri*) (Tab. 2).

Tab. 2. Mean gene diversity (He), heterozygosity (Ho) and polymorphism information content (PIC) values of the 5 studied *Dianthus* species.

Species	He	Ho	PIC
D. callizonus	0.18	0.3	0.14
D. glacialis ssp. *gelidus*	0.31	0.32	0.26
D. henteri	0.44	0.57	0.34
D. nardiformis	0.35	0.32	0.28
D. tenuifolius	0.4	0.47	0.32

High similarity among *Dianthus* species collected from Romania was observed in this study. Allelic patterns across *Dianthus* species, as revealed in Fig. 2, indicate that the mean number of different alleles was highest in *D. glacialis ssp. gelidus*, while the number of effective alleles was found to be higher in *D. tenuifolius*. The amount of alleles with a frequency equal to or higher than 5% was similar in all five species, excepting for *D. callizonus*, where it was slightly lower. Except for *D. nardiformis*, private alleles were detected in all *Dianthus* populations. The strongest genetic divergence was observed among *D. callizonus* and *D. tenuifolius*. The number of locally common alleles with a frequency higher than 5% revealed that *D. henteri* and *D. nardiformis* share almost the same alleles in most of the loci. Shannon's information index (I) reveals low levels of genetic diversity in all populations, especially in *D. callizonus* (I = 0.251). *D. henteri* was characterized by the highest genetic diversity (I = 0.626). Moreover, Shannon's index was closely related to the expected heterozygosity. A high similarity in allelic patterns was observed in case of *D. henteri* and *D. tenuifolius*.

Genetic structure and evolutionary analysis

A neighbour-joining tree showed the evolutionary relationships of taxa (Fig. 3). Thus, some of the individuals belonging to *D. henteri*, *D. glacialis* and *D. callizonus* clustered independently while an overlapping distribution was observed in case of *D. nardiformis* and *D. tenuifolius* and other individuals of previous species. This distribution was confirmed by principal coordinate analysis (PCoA). This analysis shows a relatively high diversity of patterns in *D. nardiformis* and *D. glacialis ssp. gelidus*. Consistent with the low levels of genetic diversity, a low variety of allelic profiles was observed in *D. callizonus* population (Fig. 4). The first, the second and the third principal coordinates accounted for 32.27%, 18.69% and 11.99%, respectively, explaining for 62.96 of the total genetic variation across individuals belonging to different *Dianthus* species.

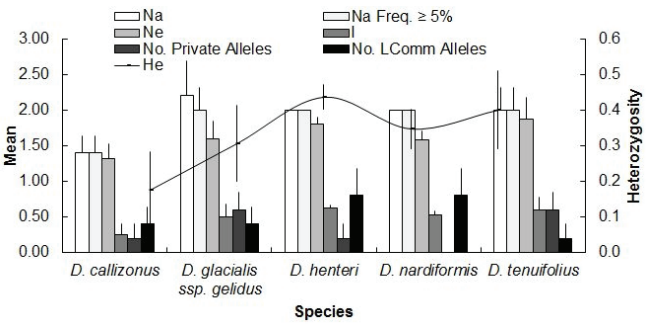

Fig. 2. Distribution of allelic patterns across *Dianthus* species. Na – number of different alleles; Na Freq ≥ 5% – number of different alleles with a frequency ≥ 5%; Ne – number of effective alleles; I – Shannon's information index; No. Private Alleles – number of alleles unique to a single species; No. LComm Alleles – number of locally common alleles with a frequency ≥ 5% found in 50% or fewer species; He – Expected heterozygosity. Error bars represent standard deviations.

Analysis of molecular variance (AMOVA) for all loci showed that the highest amount of genetic variance (51.48%) occurred among *Dianthus* species and a variance of 48.52% was found within the species. Locus-by-locus AMOVA confirmed the results of the overall AMOVA and revealed that the greatest amount of genetic variance occurred among all five *Dianthus* species (50.88%). Genetic variance found within *Dianthus* species was 49.12% (Tab. 3).

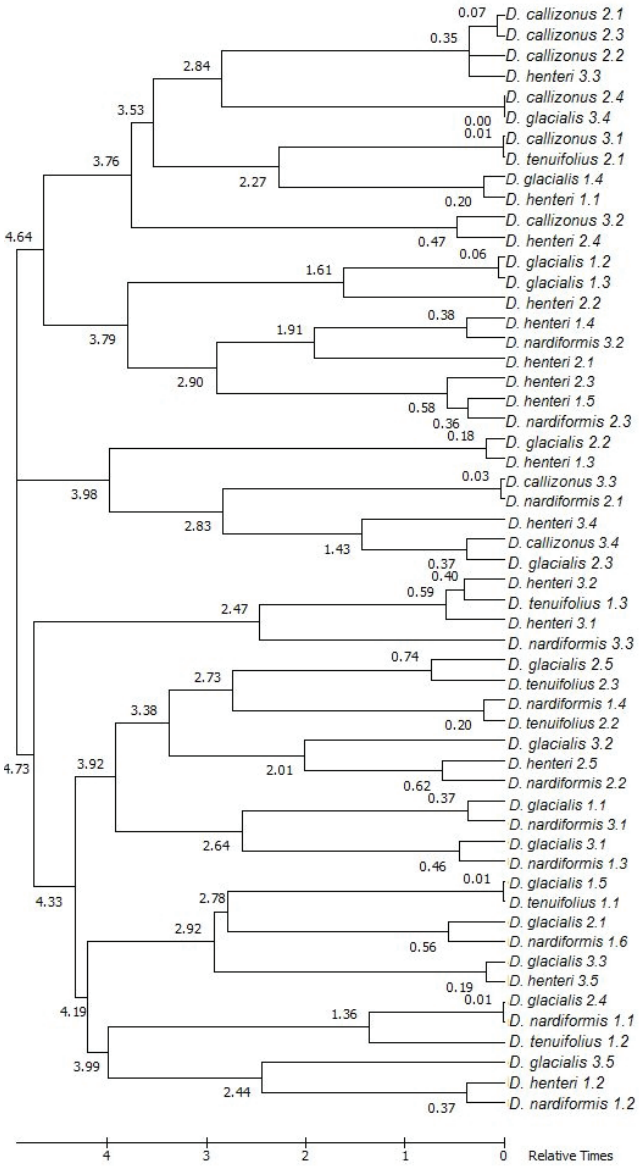

Fig. 3. Evolutionary relationships of *Dianthus* taxa in Romania.

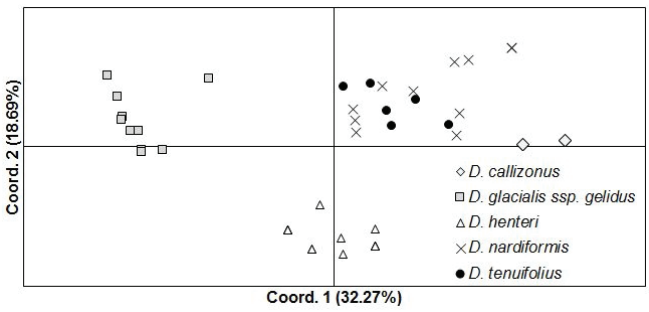

Fig. 4. A two-dimensional plot of the principal coordinate analysis (PCoA) of SSR data showing the clustering of five *Dianthus* species in Romania.

Tab. 3. Results of analysis of molecular variance (AMOVA) based on F_{ST} from data of five microsatellite loci and Gini-Simpson index of *Dianthus* species.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage variation ($p < 0.001$)
AMOVA analysis for all loci				
Among species	4	66.876	0.748	51.48
Within species	105	74.042	0.705	48.52
Locus by locus AMOVA				
Among species		83.317	0.961	50.88
Within species		94.336	0.927	49.12

Discussion

Genetic diversity of Romanian *Dianthus* taxa

Molecular investigation of *Dianthus* species from Europe has recently aroused interest in several aspects. Analysis of the genetic diversity and structure of populations of endangered, rare or endemic species of *Dianthus* are useful tools in conservation planning. Molecular analysis could aid especially in the case of plant species capable of clonal propagation as *Dianthus*. In many cases, endangered, rare or endemic species with large populations can be genetically similar or identical. In spite of the high number of individuals, the low genetic diversity variation is due to clonal multiplication or bottlenecks. Thus, data about genetic structure and variability should be useful before the development of proper conservation strategies. There are few data about molecular analysis of rare or endemic *Dianthus* species (Cristea et al. 2014, Jarda et al. 2014) despite the numerous data regarding the conservation of these species. In Romania these plant species were preserved by in vitro cultures (Butiuc-Keul et al. 2001, Miclăuş et al. 2003, Cristea et al. 2006, 2010, Marcu et al. 2006, Jarda et al. 2011, 2014). Our study revealed the genetic diversity structure of 5 endangered or endemic species of *Dianthus* found in different sites in Romania. Most of these locations have been designated NATURA 2000 Sites. By the use of SSR markers developed with the 5 primer pairs tested in this study, 10–11 alleles from a total of 23 polymorphic alleles amplified were identified in most of the species. The population of *D. callizonus* showed the lowest diversity, probably as a result of limited distribution (this is a local endemic species). According to these results, *D. callizonus* conservation should be a priority for the administration of the Piatra Craiului Natural Park. A single allele was identified in each of the loci MS-DINCARACC, MS-DINGSTA and MS-DINMADSBOX and 2 alleles were identified in loci MS-DCAMCRBSY and MS-DCDIA30.

The highest diversity was revealed in the populations of *D. glacialis* ssp. *gelidus*, especially in locus MS-DINMADS-

BOX (4 from the 6 alleles observed). This highest diversity can be explained by its larger distribution area.

Regarding the genetic diversity among populations of the same species, no differences were revealed by SSR markers tested in the present study. The highest genetic diversity was observed in *D. glacialis* ssp. *gelidus* and *D. tenuifolius* in locus MS-DINMADSBOX.

The genetic diversity is generally low in the *Dianthus* species studied here. This may be related to several factors. Mountain and alpine plants have to cope with harsh environmental conditions, e.g. short vegetation periods, unstable and low-fertility soils, desiccating winds, and high solar radiation, thus a population with low genetic diversity has limited possibilities to fight against harsh environmental conditions. In the short term, a loss of genetic diversity can reduce plant performance and population viability and may limit the potential for further adaptive evolution (Ellstrand and Elam 1993). Low genetic diversity could also be explained by the clonal propagation of many plants, as in *Dianthus* species grown in severe environmental conditions (Stöcklin 1992). Clonal propagation usually produces a rapid, but spatially limited spread of genotypes. Along an altitudinal gradient, the vegetative growth is increased, because reproduction by seeds may be hampered by the harsh alpine conditions (Young et al. 2002, Gabel et al. 2017).

The low values of gene diversity, heterozygosity and PIC found in our study are in accord with other data showing low diversity and polymorphism of *Dianthus* species from the Iberian Peninsula (Balao et al. 2010) and Iran (Farsi et al. 2013). These data seem to suggest that *Dianthus* species may be in general very young and the Romanian species are not an exception to the pattern.

It is well known that small populations isolated in fragmented habitats contain less genetic diversity than larger populations because over time, individuals will become more homozygous due to the low amounts of available genetic diversity within the population and increased inbreeding (Duminil et al. 2007). There are also complex interactions between small population size, genetic diversity and individual fitness (Aguilar et al. 2008) with several consequences, such as inbreeding depression (Jolivet et al. 2013). Human activity and land use changes was followed by small-scale fragmentation of grassland habitats in most regions of central Europe, thus small populations are facing extinction due to local maladaptation in remnant habitats (Busch et al. 2016). In Romania, populations of these endangered species of *Dianthus* seems to be also such small populations with low adaptation to the habitats. On the other hand in the case of *D. gratianopolitanus*, genetic variation within populations seems to be much more affected by population density and size than by isolation. Thus, genetic variation decreases with increasing population density across all studied populations, which may be due to the effect of gene flow within populations, which should be stronger in dense populations. Genetic variation was higher among populations of *D. gratianopolitanus* in Germany than in Switzerland. In the German region with a higher magnitude of isolation, genetic variation within populations depended on population size. In the Swiss study region, with a lower magnitude of isolation, the higher genetic variations may be attributed to the gene flow among populations even within smaller populations (Putz et al. 2015). Other studies showed that the historical landscape structure may be more important for genetic diversity than present habitat conditions. Thus, populations that were persisting in abandoned grassland fragments significantly influence the genetic variability of those species even under deteriorating habitat conditions. All of these studies may lead to the conclusion that individuals from different populations should be included in approaches to preserve the genetic variation of such plant species (Reisch et al. 2017).

Evolutionary framework and genetic variation of the studied carnations

Dianthus genus is characterized by its extensive morphological and genetic variability (Erhardt 1990, 1991, Friedman et al. 2001, Bloch et al. 2006). Many phylogenetic studies revealed that evolutionary radiations have occurred in *Dianthus* (Balao et al. 2010, Valente et al. 2010). Polyploidy, hybridization, and genome duplication, are common evolutionary forces in plants and act as potential drivers of plant radiation (Otto 2007, Paun et al. 2009, Soltis and Soltis 2009). Although interspecific hybridization is feasible in several clades of *Dianthus* (Collin and Shykoff 2003), it seems, however, that the incomplete lineage sorting and/or ancestral polymorphism processes have played a prominent roles in its recent evolutionary history (Balao et al. 2010, Valente et al. 2010). Hybridization is exceptionally high in *Dianthus* compared to many genera, partly in consequence of the recent origin of most species. High rates of hybridization have possibly contributed to fast diversification by providing evolutionary potential via lineage fusions and may explain the shared alleles.

One of the phylogenetic studies of the genus revealed that rates of diversification in Mediterranean *Dianthus* have been exceptionally high (Valente et al. 2010) and suggest that a combination of polyploidy and geographical speciation may have driven cladogenesis in the group. The major patterns of variation within and among populations of particular species on mountains have not been clearly described to date, and thus it has been difficult to discuss our results of specific case studies in relation to general patterns. However a recent study based on morphological and molecular data and analyses suggest that *Dianthus binaludensis*, distributed on Binalud Mountain, northeast Iran, also displays a local morphological divergence (Farsi et al. 2013).

In conclusion, the gene diversity is low in all of the five endemic or rare species of *Dianthus*. The genetic structure is similar in their different populations, *D. callizonus* and *D. tenuifolius* show particular sets of SSR alleles, the other 3 species of *D. glacialis* ssp. *gelidus*, *D. henteri* and *D. nardiformis* share almost the same alleles in most of the loci. Our results of evolutionary neighbour-joining analysis show overlapping distribution in the case of *D. nardiformis, D. tenuifolius* and several individuals of *D. henteri*, *D. glacialis* and *D. callizonus*.

This phenomenon could occur from the evolutionary processes in recently differentiated species such as *Dianthus* species (Balao et al. 2010, Valente et al. 2010). From these data we suggest that *D. henteri*, *D. glacialis* have not yet fully diverged. Although these two species clustered independently, several individuals clustered together with other species.

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