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GENETIC DIVERSITY ASSESSMENT OF THE ROMANIAN POPULATIONS OF *ONOSMA PSEUDOARENARIA* AND *ONOSMA ARENARIA*

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Abstract: *Onosma pseudoarenaria* Schur. (s. str.) (often cited in taxonomic literature as *O. pseudarenaria*) is a light-demanding, greyish-green, hispid-hairy species, which grows in hilly places, on dry and sandy soils. This Romanian endemic has been ascribed vulnerable species status due to its reduced number of populations, each consisting of just a few individuals, its difficult reproduction because of the sclerified fruits, and the practice of grass cutting. The genetic diversity of this species was inferred using three types of molecular markers: nrDNA sequence ITS1, cpDNA sequences and AFLP. The study also included *Onosma arenaria* Waldst. & Kit., the other representative of the group *Heterotricha* in Romania, closely related to *O. pseudoarenaria* on both morphological and taxonomic grounds. The cpDNA data showed a genetic uniformity for most of the *O. pseudoarenaria* populations, suggesting a previous genetic pattern of continuous gene flow between populations of the species. By contrast, the nuclear data inferred from AFLP showed an ongoing differentiation of the populations of the species due to the present fragmented distribution. The main conclusion of this study is the necessity to conserve all populations of *O. pseudoarenaria*, along with avoiding further decrease of the species range.

Keywords: *Onosma*, Vulnerable species, genetic diversity, AFLP, nrDNA, cpDNA.

Introduction

Onosma is a large genus which exhibits complex patterns of morphological and karyological variation, and has received controversial taxonomic treatments [11]. The genus, in Boraginaceae, tribe *Lithospermeae* Dumort., comprises *c.*150 species [23] distributed mainly in western and central Asia and in the Mediterranean region, which occur in dry, sunny, sandy, rocky and steppic habitats [13]. *Onosma* includes mainly herbaceous biennial or perennial species, morphologically well delimited, based on the hairy indumentum with each hair formed by a single large bristle growing out of the top of a tubercle, narrow anthers with sterile tips, pollen grains evidently colpate, bearing 3 pores in a single row, nutlets nearly straight and erect usually with a broad and evident basal attachment to the base of calyx, corolla-lobes that are erect or loosely recurved, filaments similar, calyx-lobes narrow and elongate, and corolla without ribs projecting between the calyx-lobes [10].

The Romanian species are: *Onosma visianii* Clementi – found in the southern and south-eastern part of the country [3]; *Onosma arenaria* Waldst. & Kit. – a species of controversial delimitation, as some authorities, including the authors of the present study, consider it confined to the Danube Delta [22], while others consider its distribution to be more widespread, in the Cluj, Hunedoara, Timişoara, Craiova, Constanţa and Iaşi regions [7]; *Onosma pseudoarenaria* Schur. (s. str.) – endemic to Romania, occurring only in a few localities in Transylvania; *Onosma taurica* Pall. – a rare species found only in rocky, calcareous areas in Timişoara and Constanţa Counties [7]; and *Onosma heterophylla* Griseb. (incl. *O. viridis* (Borbás) Jáv.) – found on sunny and rocky slopes in Cluj, Alba, Hunedoara, Sibiu, Dolj, Vâlcea and Buzău Counties [7].

Based on karyological and morphological data, three groups (originally described as sections) have been designated within *Onosma*: *Asterotricha*, *Haplotricha* and *Heterotricha*. The

only Romanian representatives of the *Heterotricha* group are *Onosma arenaria* and *O. pseudoarenaria*. It is known that *Heterotricha* includes several taxa which are morphologically very similar, but allopatric and their distribution is scattered over small sites. The classification of *Heterotricha* is very difficult, many authors [1, 14, 19, 20, 21 and 22] having attempted the task but failed to draw decisive and categorical conclusions. However, it is unanimously agreed that the group is represented by several more or less well differentiated and geographically separated clusters of populations (at least 13, of which 10 are within *O. pseudoarenaria* s.l. and three within *O. arenaria* s.l.) occurring discontinuously mainly in Central and Southern Europe, and marginally in Western and Eastern Europe. In the past, these population groups were evaluated as separate species, but subsequently some were variously reclassified as subspecies within different species by several authors, and their classification has been continuously changed over time. For example, the taxonomic status of *O. pseudoarenaria* remains controversial, some authors [3] considering it to be a subspecies of *O. arenaria*, others [2, 7, 14] regarding it as a valid species without taxonomic subordination to *O. arenaria*.

Onosma pseudoarenaria Schur. (s. str.) is a light-demanding, greyish-green, hispid-hairy species, which grows in hilly places, on dry and sandy soils. This species is a local endemic species of Transylvania, Romania, and has been ascribed vulnerable conservation status [6]. The scarce populations of the species generally comprise just a few individuals, its reproduction being impeded by its hard woody fruits and the common Romanian practice of mowing grass that prevents some individuals reaching reproductive maturity [6]. For these reasons, the molecular survey of genetic diversity in *Onosma pseudoarenaria* was considered appropriate for the optimal future conservation of this species. The study also included *Onosma arenaria*, a species that shares close taxonomic and morphological links with *O. pseudoarenaria*.

Materials and Methods

The methods used to accomplish this investigation were the sequencing of both nuclear and chloroplastic regions and genotyping of AFLP phenotypes.

Sampling strategy

In order to investigate the genetic diversity of the species *Onosma pseudoarenaria* and *Onosma arenaria*, the following populations were sampled: 2 populations of *Onosma arenaria* from the Danube Delta; 11 populations of *Onosma pseudoarenaria* (s. str.) from Transylvania, 1 population of *Onosma pseudoarenaria* (*O. pseudoarenaria* subsp. *tridentina* (Wettst.) Braun-Blanq.; syn. *Onosma tridentina* Wettst., see 14) from Deliblatska pescara, Serbia; 4 populations of *Onosma viridis* from Transylvania and Banat; 1 population of *Onosma visianii* from Biokovo, Croatia (Figure 1, Table 1). The last two species have served as outgroups in the construction of trees, *O. visianii* belonging to the *Haplotricha* group and *O. viridis* (*O. heterophylla* s.l.) to the *Asterotricha* group. Voucher specimens of all material were collected and deposited, either in the herbarium of the Institute of Botany of the Slovak Academy of Sciences (SAV) or in the private collection of the Institute of Biological Research, Cluj-Napoca.

DNA extraction

Total DNA was extracted from roughly 13 mg of silica gel-dried leaf tissue, using DNeasy 96 Plant Mini Kit (Qiagen) according to the manufacturer's protocol, except the final elution, which took place in 80 µl in order to increase the DNA concentration. Three random individuals from the total sample set were extracted twice as blind samples [2].

nrDNA and cpDNA analysis

ITS1 (Internal Transcribed Spacer 1) region belonging to the nuclear genome was amplified through PCR using the primers ITS2 and ITS5 designed by White *et al*. (1990) [24]. Amplification was performed in a 50 µl total reaction volume with 1X Taq Buffer (10X Taq Buffer, Fermentas), 2.5 mM MgCl2, 0.5 mM of each dNTP, 0.12 µM of each primer, 0.16 mg/ml BSA, 2 U of Dream Taq Polimerase (Fermentas) and 10 µl of diluted genomic DNA.

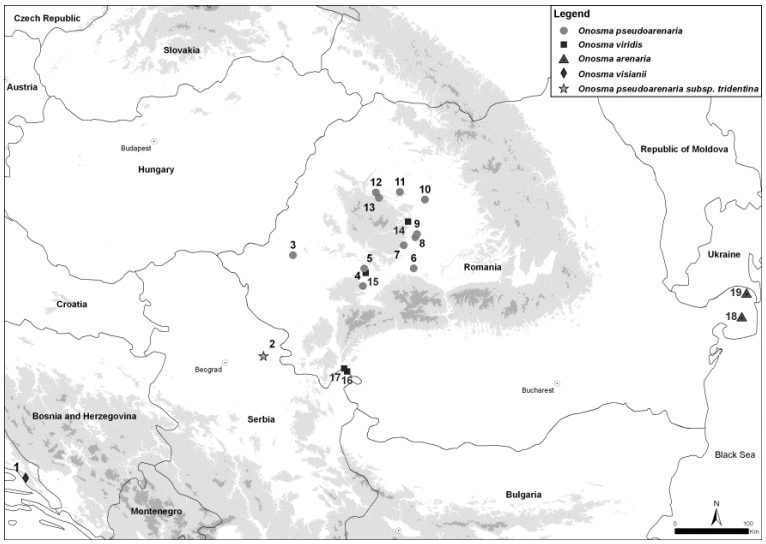

Fig. 1: Geographical origin of the sampled populations of *Onosma arenaria*, *O. pseudoarenaria*, *O. viridis* and *O. visianii*

Table 1: Species, population codes, collection localities and geographical coordinates for the sampled populations of *Onosma arenaria*, *O. pseudoarenaria*, *O. viridis* and *O. visianii*

Pop. No.	Species	Pop. Code	Locality	Coordinates (N/E)
1	*O. visianii*	BC-OVs	Biokovo, Croatia	43°18’39.2”/ 17°03’48.9”
2	*O. pseudoarenaria* subsp. *tridentina*	DEL-OP	Deliblatska pescara, Serbia	44°53’06.8”/ 21°04’45.5”
3	*O. pseudoarenaria*	P-OP	Păuliş, Arad County	46°07’28.92”/ 21°36’04.61”
4	*O. pseudoarenaria*	Go-OP	Govăjdia, Hunedoara County	45°44’04.6”/ 22°49’32.7”
5	*O. pseudoarenaria*	PH-OP	Păuliş, Hunedoara County	45°56’47.8”/ 22°51’32.7”
6	*O. pseudoarenaria*	C-OP	Cunţa, Alba County	45°55’55.7”/ 23°43’53.1”
7	*O. pseudoarenaria*	GS-OP	Galda de Sus, Alba County	46°13’19.7”/ 23°34’08.55”
8	*O. pseudoarenaria*	Cb-OP	Ciumbrud, Alba County	46°18’53.1”/ 23°46’46.5”
9	*O. pseudoarenaria*	OM-OP	Ocna Mureş, Alba County	46°21’16.28”/ 23°48’26.40”
10	*O. pseudoarenaria*	S-OP	Suatu, Cluj County	46°46’33.8”/ 23°58’30.8”
11	*O. pseudoarenaria*	Ch-OP	Chinteni, Cluj County	46°52’42.7”/ 23°31’38.4”
12	*O. pseudoarenaria*	IC-OP	Izvorul Crişului, Cluj County	46°48’55”/ 23°09’8”
13	*O. pseudoarenaria*	Sf-OP	Sfăraş, Sălaj County	46°53’00”/ 23°05’59”
14	*O. viridis*	Mdv-OV	Moldoveneşti, Cluj County	46°30’38.9”/ 23°39’29.1”
15	*O. viridis*	Dev-OV	Deva, Hunedoara County	45°53’34.0”/ 22°52’55.2”
16	*O. viridis*	Ors-OV	Orşova, Mehedinţi County	44°43’15.2”/ 22°28’15.5”
17	*O. viridis*	Or-OV	Orşova, Mehedinţi County	44°40’57.5”/ 22°31’12.6”
18	*O. arenaria*	CD-OA	Caraorman, Tulcea County	45°03’35.51”/ 29°23’28.79”
19	*O. arenaria*	LD-OA	Letea, Tulcea County	45°20’41.26”/ 29°30’57.83”

The cycling profile involved 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 45 sec at 52°C, 2 min at 72°C, with a final elongation of 10 min at 72°C.

The chloroplastic regions used in the present study were: introns *rpL16*, *trnG*, *trnL*, intergenic spacers *rpl32-ndhF*, *psbD-trnT*, *trnD-E*, *trnT-L*, *rps16-trnK* şi *trnH-psbA*. The primers and the amplification programs were taken from Shaw *et al.* (2005, 2007) [15, 16]. The PCR chemistry was the same as the one used for the ITS1 region.

PCR products were purified using the commercial kit Wizard^{R} SV Gel and PCR Clean-Up System, according to the manufacturer's protocol (Promega Corporation, USA). Sequencing was performed in a 20 µl volume using BigDye Terminator Cycle Sequencing Ready Reaction Kit, v. 3.1 according to the manufacturer's suggestions (Applied Biosystems), using the following thermal cycle parameters: 96°C, 10 sec, 35 cycles of 96°C, 10 sec, 50°C, 10 sec, 66°C, 4 min. Both DNA strands were sequenced. Excess primers and labelled ddNTPs were removed by purification with Sephadex and Sephacryl (1:1) (GE Healthcare Bio-Sciences AB). The samples were prepared prior sequencing by adding 10 µl of HiDi formamide. The samples were run on an ABI PRISM®3130 Genetic Analyzer, Applied Biosystems using a 36 cm capillary and POP-7^{TM} polymer.

Sequences were assembled and edited using BioEdit v.7.1.3 [8]. The relationships among detected nrDNA ribotypes and cpDNA haplotypes were analyzed using the program Mega 4.0 [18]. The tree construction was made through Neighbor Joining method. Bootstrap values were obtained with 1000 replicates.

AFLP protocol

The AFLP protocol followed the basic lines of Şuteu *et al.* (2011) [17] with minor modifications. The preselective and selective primers were taken from Mengoni *et al.* (2006) [12]. Amplified fragment length polymorphism (AFLP) reactions were electrophoresed on ABI PRISM®3130 Genetic Analyzer (Applied Biosystems) using 36 cm capillary and POP-7^{TM} polymer. The size-calibrated genescan files were imported into GeneMapper v.4.0 (Applied Biosystems) for scoring. Fragments within the 50–500 bp range were scored to produce a presence/absence matrix.

For the AFLP dataset, a Neighbour Joining tree was constructed with Splistree v. 4.10 [9]. Bootstrap values were obtained with 1000 replicates.

Results

nrDNA data

The ITS1 sequence (342 nucleotides) obtained revealed just one ribotype for the two populations of *O. arenaria* (GenBank accession numbers: JX267826 - JX267827). Similarly, the 12 populations of *O. pseudoarenaria* (s.l.) had one ribotype, which differed from that of *O. arenaria* by a single transversion (GenBank accession numbers: JX267828 - JX267839). The tree generated through Neighbour Joining method is shown in Figure 2.

The Neighbour Joining tree shows the grouping of *Onosma* populations according to the species criteria.

cpDNA data

The concatenated sequences of nine molecular markers: *rpL16*, *trnG*, *trnL*, *rpl32-ndhF*, *psbD-trnT*, *trnD-E*, *trnT-L*, *rps16-trnK* and *trnH-psbA*, comprised approx. 6000 nucleotides. The two populations of *O. arenaria* showed the same haplotype for all the sequenced regions (GenBank accession numbers: JX267864 - JX267865; JX267959 - JX267960; JX267997 - JX267998; JX267883 - JX267884; JX267845 - JX267846; JX267940 - JX267941; JX268016 - JX268017; JX267921 - JX267922; JX267978 - JX267979). In the case of *O. pseudoarenaria*, the cpDNA (GenBank accession numbers: JX267866 - JX267877; JX267961 - JX267972; JX267999 - JX268010; JX267885 - JX267896; JX267847 - JX267858; JX267942 - JX267953; JX268018 - JX268029; JX267923 - JX267934; JX267980 - JX267991) revealed four haplotypes:

one for the population of *O. pseudoarenaria* subsp. *tridentina* from Serbia, one for the population in Chinteni, Cluj County, one for the population in Govăjdia, Hunedoara County, while the last, identical to the *O. arenaria* haplotype, was common for all the remaining populations of *O. pseudoarenaria*. The polymorphisms that differentiate these four haplotypes are shown in Figure 3. The separation of *O. pseudoarenaria* subsp. *tridentina* from the other populations was based on the sequences belonging to *rpL16*, *trnD-E*, *psbD-trnT* and *trnL* regions. The Chinteni population was separated from the other populations on the basis of one transversion that occurred within the *rps16-trnK* region. The Govăjdia population was separated from the other populations on the basis of one transversion that occurred within the *trnT-L* region.

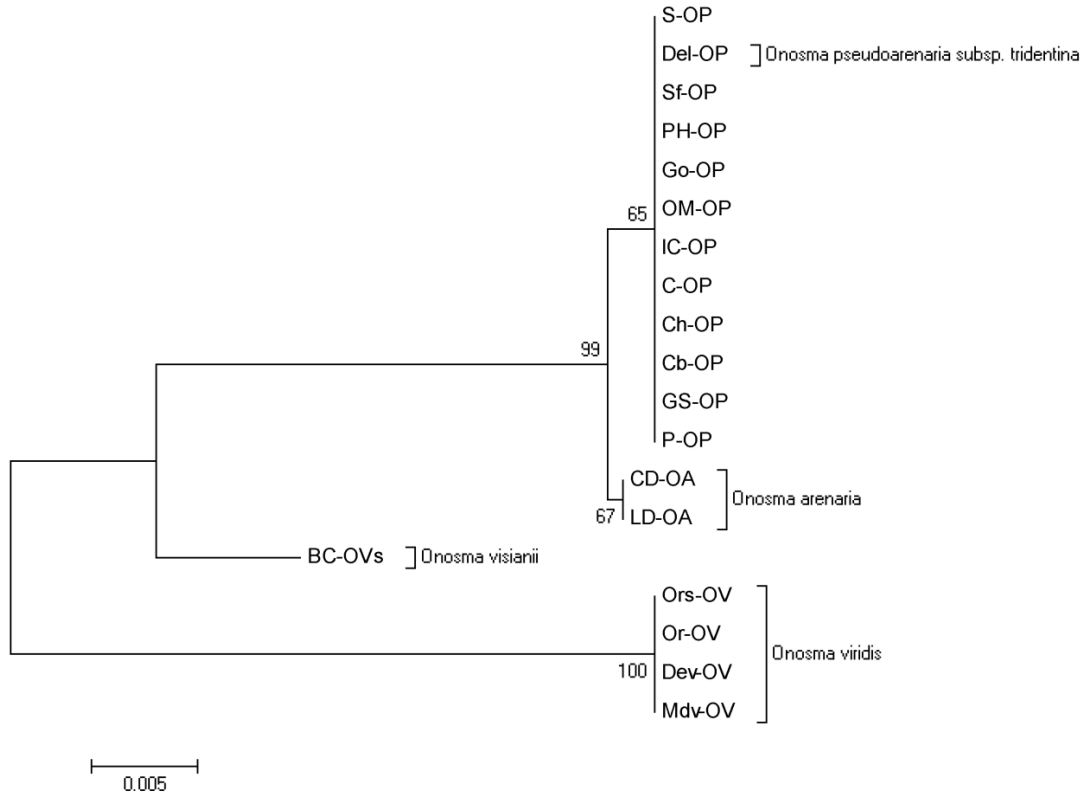

Fig. 2: Tree generated through Neighbour-Joining method based on the ITS1 ribotypes for the sampled populations of *Onosma arenaria, O. pseudoarenaria, O. viridis* and *O. visianii*. The numbers above the branches are bootstrap values (% of 1000 replicates). Populations as in Table 1.

	Absolute Position									
1	Hap 1	-	C	A	T	G	C	C	A	A	A
2	Hap 2	-	.	.	.	C	.	.	.	.	.
3	Hap 3	-	.	.	.	.	A	.	.	.	.
4	Hap 4	T	T	-	A	.	.	-	-	-	-

Fig. 3: Haplotypes and differentiating polymorphisms for *O. pseudoarenaria* (s.l.) based on nine cpDNA regions. Haplotype 1 – populations 3, 5, 6, 7, 8, 9, 10, 12, 13; Haplotype 2 – population 11; Haplotype 3 – population 4; Haplotype 4 – population 2. The numbers of the populations match Table 1.

The cpDNA pattern is presented in the tree generated through Neighbour Joining method (fig. 4).

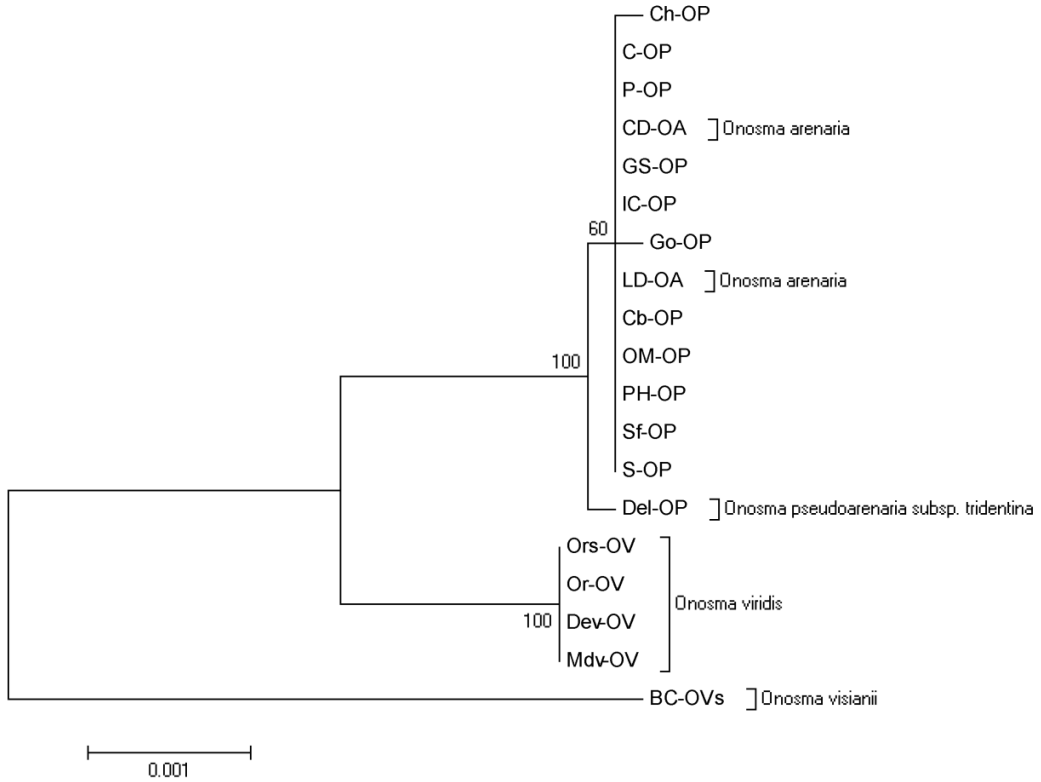

Fig. 4: Tree generated through Neighbour-Joining method based on the cpDNA haplotypes generated from nine regions for the sampled populations of *Onosma arenaria*, *O. pseudoarenaria*, *O. viridis* and *O. visianii*. The numbers above the branches are bootstrap values (% of 1000 replicates). Population codes as in Table 1.

AFLP data

Using three pairs of primers, 241 scorable fragments have been generated, of which 227 (94.19%) are polymorphic. The length of fragments ranged from 52 to 451 bp. The repeatability of AFLP results was high (100% for the overall test). The final matrix consisted of 85 individuals and 227 unambiguous polymorphic markers.

The result of the Neighbour Joining analysis is shown in Figure 5.

The Neighbor Joining tree (fig. 5) shows two major groups. The first is composed of populations of *O. visianii* and *O. viridis* that are separated from the second group by a bootstrap value of 88.4 and then they separate from each other with higher bootstrap values. The second group is composed of *O. arenaria* and *O. pseudoarenaria* populations that are very distinct, sometimes with high bootstrap values.

Discussion

At the chloroplastic level, *O. pseudoarenaria* from Romania shows genetic identity, with only two populations differing from the majority: that from Govăjdia (Hunedoara County) and that from Chinteni (Cluj County). Each of these populations is separated on the basis of a single polymorphism. The chloroplast genome is known to maintain the ancient genetic patterns for a longer period of time because of its non-recombinant nature, low mutation rate and maternal inheritance [4]. Thus, in this case, the chloroplastic uniformity might reveal a previous genetic pattern, from a period when the distribution of this species was not as fragmented as it is today. The divergence of the two populations may be due to recent genetic isolation in the case of the population from Govăjdia; and to the effect of genetic drift for the population from Chinteni. The effect of genetic drift is more powerful within populations with a small number of individuals [6], as is the case for the Chinteni population, consisting of just 1–2 individuals. The two

populations of *O. arenaria* share the same haplotype, again due to the properties of the chloroplast genome.

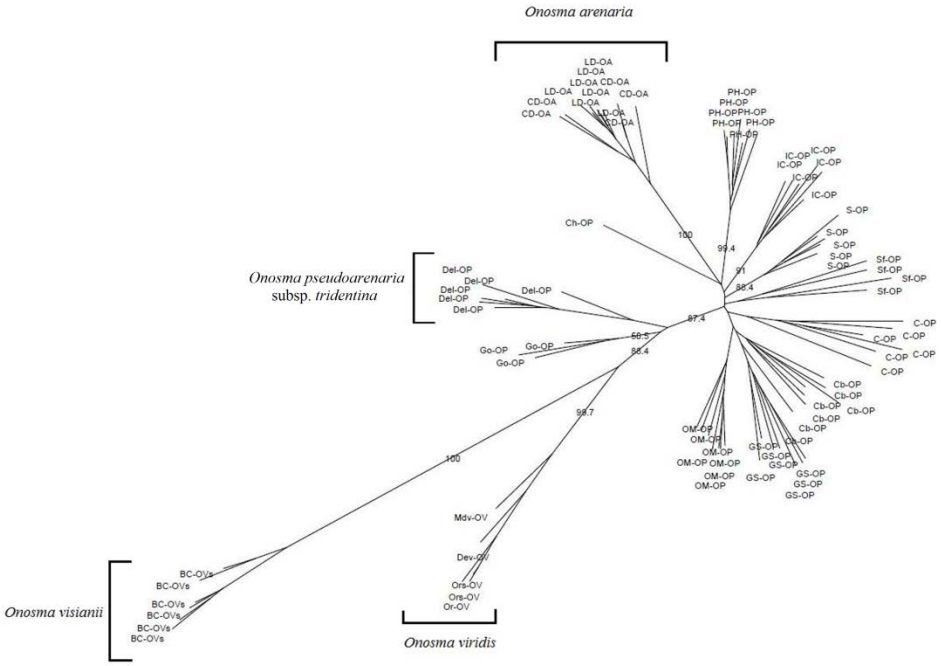

Fig. 5: Tree generated through Neighbour-Joining method based on the AFLP phenotypes for the sampled *Onosma arenaria, O. pseudoarenaria, O. viridis* and *O. visianii* populations. The numbers above the branches are bootstrap values (% of 1000 replicates). Populations codes as in Table 1.

The highest genetic diversity is revealed in the case of AFLP analysis, which scans the whole genome of the species. The Neighbor Joining tree (Fig. 5) reveals a pattern where populations are distinct and very well differentiated, sometimes with high bootstrap values. Unlike the chloroplast genome, the nuclear genome has a high mutation rate, therefore it evolves very fast and displays only recent events. Based on the nuclear genome, the *O. pseudoarenaria* populations show a recent separation as a result of distributional discontinuity.

These populations of the species are geographically separated (Figure 1), and therefore gene flow between them is interrupted. Another factor that enhances genetic differentiation is genetic drift, which acts more intensively within populations with only a few individuals, as is the case of *O. pseudoarenaria*. As for *O. arenaria*, the two populations are not so well separated, the bootstrap values that separate them being moderate. These results are not supported by the ITS1 analysis, which provided one identical ribotype for all the *O. pseudoarenaria* populations and one identical ribotype for all the population of *O. arenaria*. The explanation for this situation is that ITS1 represents only a very small part of the nuclear genome and thus is not taking into account the whole genomic variation.

Conclusions

In the case of *Onosma pseudoarenaria*, cpDNA data show a genetic identity for most populations, suggesting a previous genetic pattern of continuous gene flow between the populations of the species. By contrast, the nuclear data inferred from AFLP show an ongoing differentiation of the populations of the species due to the present fragmented distribution. This situation was not shared by *O. arenaria*. The genetic variation revealed by AFLP markers suggests that in *O. pseudoarenaria* each small population is an important element for the

diversity of the local ecosystem and a valuable genetic resource for the species. This fact imposes the necessity to conserve all the populations of *O. pseudoarenaria*, along with maintaining the whole distribution of this species.

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ESTIMAREA DIVERSITĂŢII GENETICE A POPULAŢIILOR DE *ONOSMA PSEUDOARENARIA* ŞI *O. ARENARIA* DIN ROMÂNIA

(Rezumat)

Onosma pseudoarenaria (s. str.) este un taxon endemic pentru Transilvania, heliofil, hispid, cu aspect verde cenuşiu, care vegetează în zona colinară, pe soluri uscate, nisipoase. *Onosma pseudoarenaria* are statutul de specie vulnerabilă, cele câteva populaţii componente fiind alcătuite din indivizi puţini, deoarece se înmulţeşte greu din cauza fructelor sclerificate şi a faptului că unele plante nu ajung la maturitate reproductivă din cauza cositului timpuriu. Datorită acestui statut de vulnerabilitate s-a considerat oportun studiul diversităţii genetice a acestei specii, în vederea unei conservări viitoare optime. Studiul a inclus şi *O. arenaria*, care prezintă strânse legături taxonomice, morfologice şi ecologice cu *O. pseudoarenaria*, iar drept outgroup-uri s-au utilizat *O. viridis* şi *O. visianii*. Metodele folosite au fost secvenţierea regiunii nucleare ITS1, secvențierea a nouă regiuni cloroplastice şi tehnica AFLP. În cazul regiunii nucleare ITS1 s-au obținut două ribotipuri, unul corespunzător pentru *O. arenaria* și unul corespunzător pentru *O. pseudoarenaria*, cele două ribotipuri diferențiindu-se printr-o singură mutație punctiformă. În cazul regiunilor cloroplastice s-a obținut un singur haplotip pentru cele două populații de *O. arenaria* și patru haplotipuri pentru populațiile de *O. pseudoarenaria*. Haplotipul dominant identificat pentru *O. pseudoarenaria* este identic cu cel evidențiat în cazul speciei *O. arenaria*, celelalte trei haplotipuri întâlnindu-se la populațiile din Chinteni, județul Cluj, Govăjdia, județul Hunedoara și Deliblatska pescara, Serbia. Populațiile din Chinteni și Govăjdia se diferențiază fiecare de haplotipul dominant printr-o singură mutație, ceea ce nu le separă clar de celelalte populații în cadrul arborelui generat prin metoda Neighbour-Joining. Populația din Serbia se separă clar în cadrul arborelui generat, pe baza a opt mutații punctiforme survenite în regiunile *rpL16*, *trnD-E*, *psbD-trnT* și *trnL*, dar această separare este previzibilă având în vedere atât distanța geografică dintre această populație și celelalte populații colectate din România cât și faptul că ea reprezintă subspecia *tridentina* a speciei *O. pseudoarenaria*. Aşadar rezultatele arată o similaritate genetică la nivel de genom cloroplastic, sugerând existenţa unui flux genetic ancestral continuu. Pe de altă parte, arborele generat pe baza datelor AFLP arată însă o separare genetică certă, cu valori mari de bootstrap, a tututor populaţiilor speciei *O. pseudoarenaria*. Acest rezultat provenit în special din analiza genomului nuclear sugerează un flux genetic întrerupt și o izolare genetică recentă a acestor populații. Concluzia studiului este necesitatea conservării tuturor populaţiilor de *O. pseudoarenaria* în scopul menţinerii integrale a variabilităţii genetice a speciei şi evitarea reducerii arealului acestei specii.

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