Hop and Medicinal Plants, Year XXXII, No. 1-2, 2024
ISSN 2360 – 0179 print, ISSN 2360 – 0187 electronic

Molecular Analysis of the Species *Dracocephalum moldavica* L.

SIMEA Ştefania-Alexandra[1], FLORIAN Teodora[1], BUTIUC-KEUL Anca[2], DUDA Matei Marcel[1]

[1]University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Faculty of Agriculture, Department of Crop Science, 3-5 Calea Mănăştur, 400372, Cluj-Napoca, Romania

[2] Babeş-Bolyai University, Faculty of Biology and Geology, Department of Molecular Biology and Biotechnology,
M. Kogălniceanu Street, No 1, 400084 Cluj-Napoca, Romania
*Correspondence: stefaniasimea@gmail.com

Abstract: There is a growing global trend in the consumption of cultivated medicinal and aromatic plants and an increasing demand for medicinal plants for the production of herbal medicines, health products, dietary supplements, and cosmetic products. The raw plant material from medicinal plant crops is insufficient to meet the needs of this industry. There is a preference for cultivated plant material because most pharmaceutical companies prefer raw materials that meet the required quality standards. Therefore, expanding the cultivation of medicinal plant and introducing new species into cultivation is becoming an important objective. These efforts are complemented by other advantages of cultivation (e.g., modern mechanized agricultural techniques, irrigation, fertilization, and mechanized harvesting). Molecular characterization of the species *Dracocephalum moldavica*, was focused on analysing the genetic structure of a cultivated plant population. Genetic analysis can provide important data regarding intra- and interpopulation genetic polymorphism, closely correlated with the adaptive capacity of the species to different pedo-climatic conditions, as well as the biosynthetic capacity of pharmacologically active compounds. This enables the accurate identification and selection of new valuable genotypes. Molecular analysis can be performed using DNA molecular markers, among which SSR (simple sequence repeats) and ISSR (inter simple sequence repeats) markers are frequently used due to their high reproducibility, relatively low cost, and avoidance of radioactivity (as is the case with other molecular markers). In this study, ISSR markers revealed genetic polymorphism of cultivated plants.

Keywords: genotypes, growing, medicinal plants, molecular analysis, molecular markers.

Introduction

Dracocephalum moldavica L., traditionally known as mătăcină, bee balm, monastery basil, dragonhead, matica (from Old Slavic = queen of the bees) balm, is a species belonging to the *Lamiaceae* family, widely recognized for its aromatic properties (Simea et al., 2018; Simea et al., 2023). It is an annual herbaceous species that can be found in temperate regions, originating from Central Asia and naturalized in Europe. In Romania, the species is rarely found in the wild flora and is only present in limited areas in the eastern, northwestern, and southern parts of the country. The plant has thin, brown roots; lower leaves with petioles and crenate-toothed margins; and upper leaves that are sessile with serrated-spiny edges. The stem is upright, reddish, and strongly branched at the base. The corolla of the flowers is usually purple-blue and, less commonly, light blue or white, while the fruits are brown, ovoid, and tetranuclear (Simea et al., 2023). It blooms in July and forms fruits in August, containing flavonoids, terpenes, proteins, amino acids, polypeptides, and 16 amino acids, 8 of which are essential. It also contains 1.03% essential microelements, such as Fe, Cu, Mn, and Sr. The tincture from the dried plant has been used for centuries in traditional Uighur medicine to treat heart diseases, hypertension, angina, tracheitis, atherosclerosis, neuralgia, migraines, headaches, and toothaches. Extracts of *Dracocephalum* possess sedative, analgesic, wound-healing, and antispasmodic activity (Sultan et al., 2008).

This species is used in the manufacture of perfumes and soaps due to its high content of citral and geraniol. It is also used to flavour compotes and jams, spirits, syrups, and canned fish. Industrially, vitamin A can be obtained from citral. In infusions or teas, the plant is used to soothe colic and nervousness, induce restful sleep, and reduce nausea in pregnant women (Muntean et al., 2016).

To date, numerous studies have demonstrated that *Dracocephalum moldavica* L. possesses a diverse range of therapeutic effects**,** such as antioxidant, sedative, antinociceptive, antimicrobial, cardiovascular protective, antidepressant, anti-inflammatory, anticancer, anti-complement, antifungal, and other pharmacological activities. In addition, *Dracocephalum moldavica* not only holds significant medicinal value in traditional medicine but also has unique applications in food industry, biological pesticides, cosmetics, and more. Interestingly, in the context of food,

Dracocephalum moldavica leaves can be used not only for pressed snacks but also to make bread by adding dried or powdered leaves to wheat flour (Dastmalchi et al., 2007).

Dracocephalum moldavica is an annual herbaceous cultivated species, rarely found in the wild, with a height of 30-70 cm. Distinctly four-angled, sometimes with reddish hues and hairy. The basal branches, nearly as long as the main stem, are erect. It is erect and richly branched from the base, emitting a pleasant lemon scent (Muntean et al., 2016).

Herba Dracocephali consists of branched stems with erect branches, elongated-lanceolate leaves, deeply serrated and crenate margins, glabrous or very short-haired, glandular-dotted on the underside, and truncate or narrowly cuneate at the base. The leaves are 1.5-7 cm long and 0.7-2 cm wide. The flowers are grouped in whorls of 6-10, with white or blue-violet corollas. The raw material consists of the leafy aerial parts, harvested before full flowering. The scent is pleasant, lemony, and the taste is aromatic, slightly bitter (Muntean et al., 2016).

Materials and methods

1. DNA Isolation

DNA was isolated from leaves (to avoid endangering the natural gene pool) collected from plants in their crop using the method developed by Doyle and Doyle 1987. Several individuals from each species and population were analysed as previously described.

The basic steps in DNA extraction are as follows: cell wall disruption by grinding or sonication, removal of lipid membranes using a detergent, removal of proteins by adding a protease, and DNA precipitation with an alcohol. Finally, the DNA is solubilized in an alkaline solution or ultrapure water.

CTAB DNA Isolation Doyle and Doyle 1987

DNA isolation was performed at room temperature using 100-200 mg of plant material, which was placed in 1.5 ml Eppendorf tubes.

Work was carried out using sterile tips, Eppendorf tubes, and gloves.

Experimental Protocol

Homogenization: 100-200 mg of plant material is thoroughly homogenized with 100 µl of CTAB buffer, then 400 µl of CTAB buffer and PVP are added.

RNAse Treatment: Add 2 µl of RNase per sample and incubate in a shaking block for at least one hour at 65°C.

Centrifugation: Centrifuge for 10 minutes at 14,000 rpm at room temperature.

Phase Separation: Transfer 500 µl of the supernatant to new Eppendorf tubes and add 200 µl of a chloroform/isoamyl alcohol (24/1) mixture. At this stage, the solution can be stored overnight in the refrigerator. Mix vigorously for 30 seconds.

Centrifugation: Centrifuge at 4°C as described in step 3.

Isopropanol Precipitation: Pipette 400 µl (2 x 200 µl) into new Eppendorf tubes and add 200 µl of isopropanol [1:1] [v/v]. Gently mix by hand and let sit for a few minutes.

Centrifugation: Centrifuge for 10 minutes at 4°C and 13,500 rpm.

Pellet Washing: Remove the supernatant and wash the pellet with 1 ml of 70% ethanol until the pellet separates.

Centrifugation: Centrifuge for 10 minutes at 4°C and 13,500 rpm. Remove the supernatant.

Drying and Rehydration: Allow the pellet to air dry at room temperature until it becomes transparent, then add 50 µl of pure water and leave overnight at 4°C.

Assessment of DNA Purity

The most commonly used method for assessing DNA purity is the spectrophotometric method. It is known that the vast majority of biological substances have characteristic absorbance in the ultraviolet (UV) range. The absorbance band of biological molecules in the UV range is quite broad, between 200-400 nm. However, the absorption peaks are fairly characteristic of different classes of substances. Proteins, which are the major contaminants in DNA solutions, have an absorbance peak at 280 nm. Nucleic acids have an absorbance peak at 260 nm.

According to Beer's Law, there is a linear relationship between the concentration of a compound and its absorbance at a specific

wavelength. Based on this principle, DNA concentration is calculated, and purity is assessed relative to proteins.

In practice, the ratio of absorbance at 260 nm to 280 nm (A260/A280) is calculated. DNA is considered sufficiently pure if the ratio falls between 1.8 and 2.0. Values higher than 2.0 indicate contamination with RNA, while values lower than 1.8 suggest contamination with proteins or phenol.

Calculation of DNA Concentration

The most accurate and straightforward method is spectrophotometry. Based on Beer's Law, concentrations corresponding to optical densities (absorbances) of pure standard DNA solutions at 260 nm have been calculated. Thus, an optical density (OD) of 1 corresponds to 50 µg/ml double-stranded DNA, 33 µg/ml single-stranded DNA, 40 µg/ml RNA, and approximately 20 µg/ml single-stranded oligonucleotides (Doyle and Doyle, 1987).

Modern spectrophotometers automatically calculate DNA concentration based on readings at 260 nm and 280 nm using the Warburg and Christian formula. According to this formula:

$$C_{\text{nucleic acid}} = 62.9 \times A_{260} - 36.0 \times A_{280}\ (\mu g/ml)$$

Sometimes, the quantities of DNA requiring concentration determination are very small, or the contamination of DNA is significant enough to interfere with UV readings. Therefore, alternative methods, such as the Saran Wrap method or minigel method, are often preferred. These methods allow the estimation of very small quantities, down to 1-5 ng of DNA.

Principle of SSR and ISSR Marker Analysis and Reaction Mix

The analysis was carried out by amplifying DNA using PCR with specific programs tailored to the primers used. For SSR marker analysis, 6 primers were utilized: IR2, IR25, IR32, IR38, IR41, and IR91 (Harris and Klooster, 2011). For ISSR marker analysis, 9 primers were employed: UBC808, UBC809, UBC811, UBC812, UBC818, UBC855, UBC856, UBC857, and UBC873 (Bahraminejad et al., 2012; Rostami-Ahmadvandi et al., 2013). The characteristics of the primers used are listed in Table 1.

Table 1

Characteristics of primers used in SSR and ISSR analysis

Name	Sequence (5′→3′)
SSR	
IR2	F: GCATTGATTGGAAACAACTACCCCT R: TGGATCGTGGATTCGAGAACGG
IR25	F: TGGGAGGAAGTGGGGTTTGAGA R: CCCTGTCAAGTTGTTGGGGTCA
IR32	F: GTAGTTGATTGCGGCCCTTCAG R: AGGTGATCAGAGACAACTCCAG
IR38	F: ACCTCCTTCTCTCCGGCTAAGG R: CAGTCACCGTTCCGGTATCTCG
IR41	F: TCAAGATAGTTCTTTGCCTGAATGGA R: CGGTTTACGCAGTCCTCTGTGA
IR91	F: ATTGGGCCTTCCTTTTGCCCTT R: ATGTCTCGTCGTGCAGTCCTGA
ISSR	
UBC808	$(AG)_8C$
UBC809	$(AG)_8G$
UBC811	$(GA)_8C$
UBC112	$(GACA)_4$
UBC818	$(CA)_8G$
UBC855	$(AC)_8YT$
UBC856	$(ACAC)_4YG$
UBC857	$(AC)_8T$
UBC873	$(ATG)_6$

PCR Reaction Mix/The PCR reaction mix per sample contains: 0.2 mM $MgCl_2$, 200 μM of each dNTP, 1 μM of each primer, 1.5 U of Taq polymerase (Fermentas), 25 ng of genomic DNA, the final volume of the reaction is 25 μl.

Amplification was carried out using a Palm-Cycler Corbett thermocycler. The amplification programs were optimized for the taxa analysed in the study and are detailed in Table 2. The amplicons were migrated in a 1.5% agarose gel stained with ethidium bromide and visualized under UV light.

Table 2

Amplification programs used for SSR and ISSR testing

Program	The initial denaturation	Stages (30 cycles)	Final elongation	Markers
1	94°C 4 min	Denaturation-94°C, 30 s Primer annealing-55°C, 30 s Elongation-72°C, 30 s	72°C 8 min.	SSR
2	94°C 4 min	Denaturation-94°C, 30 s Primer annealing-45°C, 30 s Elongation-72°C, 40 s	72°C 8 min.	ISSR

Results and Discussion

Isolation of Genomic DNA

In our experiments, total genomic DNA was used. Due to its large molecular weight, it is located near the start of migration on the electrophoresis gel. The electrophoretic patter of DNA isolated from different individuals of *Dracocephalum moldavica* is shown in Figure 1. From the electrophoretic patter of the bands corresponding to genomic DNA, it is observed that there is a large amount of DNA, and its quality is very good, with no degradation.

To calculate the purity of the isolated DNA, absorbance readings at 260 and 280 nm were taken with a spectrophotometer. The ratio of these absorbances indicates the DNA purity. In our case, this ratio ranged between 1.7 and 1.8, with an ideal value being 1.8. The concentrations obtained ranged between 30-60 μg DNA/ml. Given the concentrations obtained, which were too high for subsequent PCR amplifications and especially to avoid contamination of the DNA stock, 3% solutions were prepared. These were stored in the freezer at -20°C.

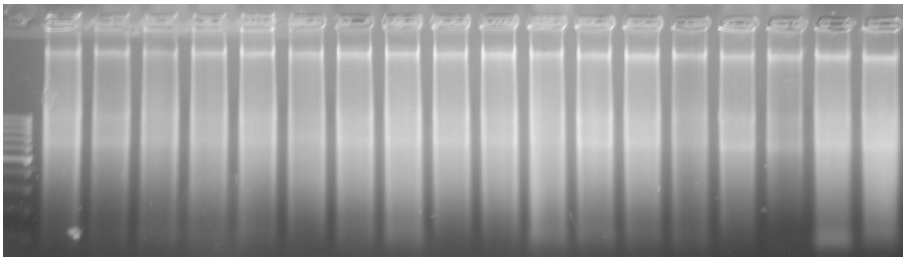

Figure 1. DNA isolated from individuals of *Dracocephalum moldavica* (1-molecular weight marker, 2-8-different plants from A1 group, 9-12-different plants from A2 group, 13-16- different plants from B1 group, 17-19- different plants from B2 group) 1% agarose gel separation, ethidium bromide visualization.

Analysis of SSR Markers

Given that the SSR markers analysed are not specific to the *Dracocephalum* genus, as previously mentioned, and there are no specific markers developed for this genus, the preliminary results we obtained are not as desired. The annealing temperature of the primers, indicated in other studies conducted on *Lamiaceae* species, was 55°C. However, in our case, clear bands were not obtained during amplification. We believe that further experiments are necessary, where the annealing and elongation temperatures are adjusted, since we do not know the size of the amplicons that might be generated.

Analysis of ISSR Markers

Amplification of DNA with the 9 primers specific to ISSR markers revealed a varied polymorphism in *Dracocephalum moldavica* individuals. An annealing temperature of 45°C was used for the primers, which was not optimal for some of the primers and requires further optimization. However, some primers generated well-defined fragments, allowing for a preliminary analysis of the plants. Primers UBC809, UBC811, and UBC856 did not produce any fragments during amplification. Therefore, reaction conditions will be modified, especially for these primers, to determine whether this was the cause or if these primers do not find complementarity with the genome of this plant species and thus cannot be used for molecular analysis of this species. Primers UBC808, UBC812, UBC818, and UBC855 produced polymorphic patterns, and it was checked whether these patterns are consistent after optimizing the amplification reactions. Primer UBC857 produced a single band, this marker being non-polymorphic.

The following sections will present only a few electrophoretic aspects to exemplify either the presence of genetic polymorphism (Figure 2) or its absence (Figure 3) with some of the primers used in the study. The number of bands highlighted by ISSR markers is shown in Table 3. It can be observed that the highest number of bands was obtained with primers UBC812, UBC818, and UBC855, specifically 7 bands, which were present only in some of the plants, indicating that the markers are polymorphic. The lowest number of bands was obtained with primer UBC857, yielding a single band present in all plants. Regarding primer UBC808, it is noted that it

highlighted polymorphism in plants from groups A1 and B2. Primer UBC812 showed polymorphism only in plants from groups A1 and A2. Primer UBC818 generated polymorphic bands in all plants from all groups except those in group A2, while primer UBC855 produced polymorphic patterns in plants from all groups except those in group B2.

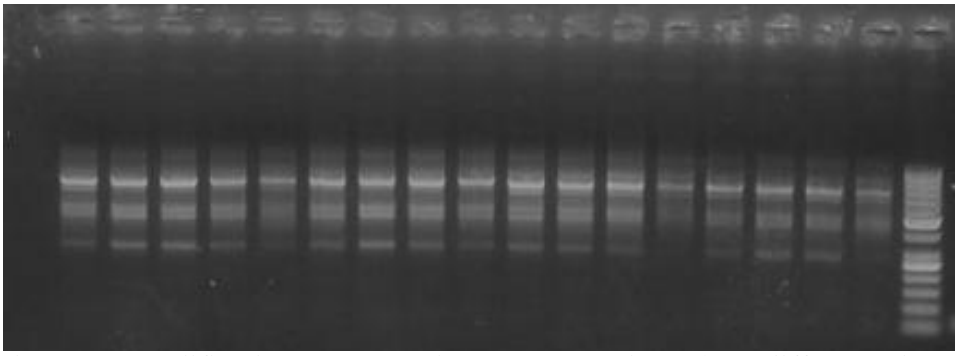

Figure 2. Amplification pattern using UBC855 primer (1-7- different plants from A1 group, 8-11- different plants from A2 group, 12-15- different plants from B1 group, 16-18- different plants from B2 group, 19- Fermentas molecular weight marker, SM1133). 1% agarose gel separation, ethidium bromide visualization.

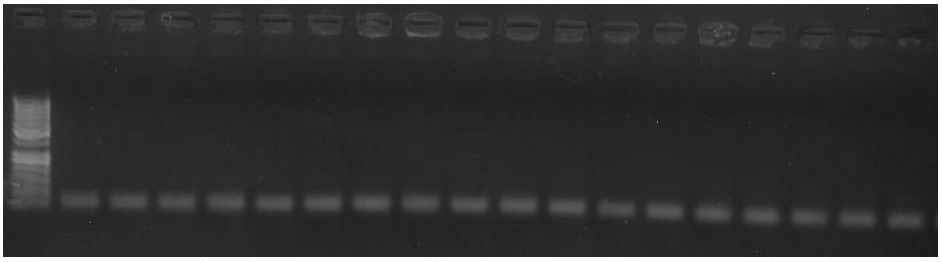

Figure 3. Amplification pattern using primer UBC857 (1-molecular weight marker Fermentas, SM1133, 2-8- different plants from A1 group, 9-12- different plants from A2 group, 13-16- different plants from B1 group, 17-19- different plants from B2 group). 1% agarose gel separation, ethidium bromide visualization.

Table 3

Polymorphism of ISSR markers in *Dracocephalum moldavica* plants

Plants	ISSR markers (band no.)								
	UBC808	UBC809	UBC811	UBC812	UBC818	UBC855	UBC856	UBC857	UBC873
A1.1	3	0	0	6	5	0	0	1	0
A1.2	3	0	0	7	5	6	0	1	0
A1.3	4	0	0	7	6	5	0	1	0
A1.4	3	0	0	9	6	5	0	1	0
A1.5	2	0	0	6	6	5	0	1	0
A1.6	4	0	0	6	6	7	0	1	0
A1.7	4	0	0	7	5	6	0	1	0
A2.1	2	0	0	6	5	6	0	1	0
A2.2	2	0	0	6	5	6	0	1	0
A2.3	2	0	0	7	5	5	0	1	0
A2.4	2	0	0	6	5	7	0	1	0
B1.1	2	0	0	7	6	7	0	1	0
B1.2	2	0	0	7	7	7	0	1	0
B1.3	2	0	0	7	7	4	0	1	0
B1.4	2	0	0	7	6	6	0	1	0
B2.1	3	0	0	6	6	5	0	1	0
B2.2	2	0	0	6	7	5	0	1	0
B2.3	3	0	0	6	7	5	0	1	0

Lamiaceae is the sixth largest angiosperm family and contains more than 7000 species distributed all over the world. This family has many phylogenetically unresolved genera and therefore many species are of not determined relationship (Li et al., 2016).

Thus, molecular markers are valuable tools for genetic characterization of plant species and for analysis of the genetic polymorphism is their population in order to identify valuable genotypes producing compounds with different applications. As for example Song et al. (2010), reported high genetic similarity reflecting low genetic diversity in *Salvia miltiorrhiza* using SRAP and ISSR

markers. Indeed, Zhang et al. (2013) reported the high importance of DNA genetic diversity of *S. miltiorrhiza* using ISSR marker in plant breeding programs.

Conclusions

- The ISSR markers analysed revealed genetic polymorphism in the *Dracocephalum moldavica* plants studied.
- The highest polymorphism was observed with markers generated using primers UBC808, UBC812, UBC818, and UBC855.
- Markers generated with primers UBC818 and UBC855 revealed genetic polymorphism, including inter-group polymorphism.
- The marker identified with primer UBC857 is non-polymorphic.

Acknowledgements
This research was conducted as part of my doctoral thesis, "Studies on the Biology, Cultivation Technology, and Utilization of *Dracocephalum moldavica* L." I wish to express my gratitude to those who contributed to its completion.

References

Bahraminejad A., Mohammadi-Nejad G., Kadir M.A., Yusop M.R.B., 2012, Molecular diversity of Cumin (*Cuminum cyminum* L.) using RAPD markers. AJCS 6(2):194-199.

Dastmalchi K., Dorman H.J.D., Oinonen P.P., Darwis Y., Laaksi I., Hiltunen R., 2007, Chemical Composition and in Vitro Antioxidative Activity of a Lemon Balm (*Melissa Officinalis* L .) Extract. LWT - Food Sci. Technol., 41:391–400.

Doyle J., Doyle J.L., 1987, A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19:11-15.

Harris, E.S.J., Klooster, M.R., 2011, Development of microsatellite markers for the medicinal plant *Isodon rubescens* (Lamiaceae) and related species. Amer J Bot. e293-e295.

Li B., P.D. Cantino, G.R. Olmstead, C.G.L.C. Bramley, C.L. Xiang, Z.H. Ma, Y.H. Tan, D.X. Zhang, 2016, A large-scale chloroplast phylogeny of the Lamiaceae sheds new light on its subfamilial classification. Sci. Rep. 6:34.

Rostami-Ahmadvandi H., Cheghamirza K., Kahrizi D., Bahraminejad S., 2013. Comparison of morpho-agronomic traits versus RAPD and ISSR markers in order to evaluate genetic diversity among *Cuminum cyminum* L. accessions. AJCS 7(3):361-367.

Muntean L.S., Tămaş M., Muntean S., Muntean L., Duda M.M., Vârban D.I., Florian S., 2016, Tratat de plante medicinale cultivate şi spontane, ediţia a II-a. Ed. Risoprint Cluj-Napoca.

Simea Ş., Duda M.M., Gheţe A.B., Mureşan C., Crişan I., 2018, The importance and use of the species *Dracocepahlum moldavica* L. Hop and Medicinal Plants, Year XXVI, No. 1-2, Ed. AcademicPres Cluj-Napoca, ISSN 2360–0179 print, ISSN 2360–0187 electronic, p.39-43.

Simea Ş., Ielciu I., Hanganu D., Niculae M., Pall E., Burtescu R.F., Olah N.-K., Cenariu M., Oniga I., Benedec D., 2023, Evaluation of the Cytotoxic, Antioxidative and Antimicrobial Effects of *Dracocephalum moldavica* L. Cultivars. Molecules 28:1604. https://doi.org/10.3390/ molecules28041604.

Sultan A., Bahang, Aisa H.A., Eshbakova K.A., 2008, Flavonoids from *Dracocephalum Moldavica* L. *Chem. Nat. Compd.* 2008, *44* (3):366–367.

Song Z., Li X., Wang H., Wang J., 2010, Genetic diversity and population structure of *Salvia miltiorrhiza* Bge in China revealed by ISSR and SRAP. Genetica, 138(2):241-9.

Zhang Y., Li X., Wang Z., 2013, Diversity evaluation of *Salvia miltiorrhiza* using ISSR markers, Biochem Genet 51(9-10): 07-21.