



USE OF SEQUENCING METHODS FOR SPECIES IDENTIFICATION EXEMPLIFIED BY PHYLOGENETIC RELATIONSHIPS WITHIN GENUS *HEDYSARUM* L.

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At the moment, a relevant objective in pharmacognosy, is the use of all kinds of the DNA analysis methods for identifying plant materials, detecting counterfeits, genetically modified crops and products.

The aim of the research is to study the possibility of using molecular genetic research methods in the analysis of the genus *Hedysarum* L., for the identification of medicinal plant materials. This article presents the results of the application of molecular genetic research methods in the analysis of the genus *Hedysarum* L. in the flora of the North Caucasus.

Materials and methods. The study material was the samples of the genus *Hedysarum* L. species collected in the North Caucasus: *Hedysarum caucasicum* M. Bieb. (in the fruiting phase in the territory of the Karachay-Cherkess Republic); *Hedysarum grandiflorum* Pall. (in the fruiting phase in the Volgograd region); *Hedysarum daghestanicum* Rupr. ex Boiss. (in the flowering phase in the Republic of Dagestan). Sequencing of the ITS1-5.8S-ITS2 marker region of gene 5.8S by the RNA ribosome was carried out according to the Sanger method on the AbiPrism 3130 genetic analyzer at the laboratory of biosystematics and cytology of Komarov Botanical Institute of the Russian Academy of Sciences.

Results. Based on a comparative study of the marker region of the nuclear ribosomal gene 5.8S rRNA, marker nucleotide substitutions of *Hedysarum caucasicum* M. Bieb., *Hedysarum daghestanicum* Rupr. ex Boiss., *Hedysarum grandiflorum* Pall., have been identified. The most probable secondary structure of 5.8S rRNA has been constructed. It has been shown that based on the analysis performed, it is possible to predict additional raw material sources of mangiferin and other groups of xanthenes using the molecular data exemplified by the *Obscura* section.

Conclusion. Based on the data obtained, it can be concluded that the morphological classification of the genus *Hedysarum* L. can be confirmed within the *Obscura* section.

Keywords: *Hedysarum*; *Hedysarum caucasicum*; *Hedysarum daghestanicum*; *Hedysarum grandiflorum*; sequencing; 5.8S rRNA gene

Abbreviations: rRNA – ribosomal ribonucleic acid; tRNA – transfer ribonucleic acid; RNase – ribonuclease; CTAB – cetyltrimethylammonium bromide; DNA – deoxyribonucleic acid.

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ИСПОЛЬЗОВАНИЕ МЕТОДОВ СЕКВЕНИРОВАНИЯ ДЛЯ ИДЕНТИФИКАЦИИ ВИДОВ НА ПРИМЕРЕ ФИЛОГЕНЕТИЧЕСКИХ СВЯЗЕЙ В ПРЕДЕЛАХ РОДА *HEDYSARUM* L.

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Актуальной задачей в фармакогнозии на данный момент является применение всевозможных методов ДНК-анализа для идентификации растительного сырья, выявления фальсификатов, генетически модифицированных сельскохозяйственных культур и продуктов.

Цель. Изучить возможность применения молекулярно-генетических методов исследований при анализе рода *Hedysarum* L. для идентификации лекарственного растительного сырья. В данной статье представлены результаты применения молекулярно-генетических методов исследования при анализе рода *Hedysarum* L. флоры Северного Кавказа.

Материалы и методы. Материалом для исследования послужили образцы видов рода *Hedysarum* L., собранные на территории Северного Кавказа: *Hedysarum caucasicum* M. Bieb. (фаза плодоношения на территории Карачаево-Черкесской Республики); *Hedysarum grandiflorum* Pall. (фаза плодоношения в Волгоградской области); *Hedysarum daghestanicum* Rupr. ex Boiss. (фаза цветения в Республике Дагестан). Секвенирование маркерного участка ITS1-5.8S-ITS2 гена 5.8S рибосомой РНК проводили по методу Сэнгера на генетическом анализаторе AbiPrism 3130 на базе лаборатории биосистематики и цитологии Ботанического института имени В.Л. Комарова РАН.

Результаты. На основе сравнительного изучения маркерного участка ядерного рибосомного гена 5.8S рРНК были идентифицированы маркерные нуклеотидные замены *Hedysarum caucasicum* M. Bieb., *Hedysarum daghestanicum* Rupr. ex Boiss., *Hedysarum grandiflorum* Pall. Была построена наиболее вероятная вторичная структура 5.8S рРНК. Показано, что на основании проведенного анализа можно составить прогноз дополнительных сырьевых источников мангиферина и других групп ксантонов при использовании молекулярных данных, на примере секции *Obscura*.

Заключение. На основании полученных данных можно сделать заключение о том, что в пределах секции *Obscura* можно подтвердить морфологическую классификацию рода *Hedysarum* L.

Ключевые слова: *Hedysarum*; *Hedysarum caucasicum*; *Hedysarum daghestanicum*; *Hedysarum grandiflorum*; секвенирование; ген 5.8S рРНК

Список сокращений: рРНК – рибосомная рибонуклеиновая кислота; тРНК – транскрипционная рибонуклеиновая кислота; РНКазы – Рибонуклеаза; СТАВ – гексадецилтриметиламмоний бромид; ДНК – дезоксирибонуклеиновая кислота.

INTRODUCTION

Alongside with the known methods of the pharmacognostic analysis, lately, a promising molecular genetic research method has been introduced [1–5, 20]. Molecular pharmacognosy methods occupy a fairly stable position in the preparation of regulatory documents for medicinal plant raw materials, as well as in the study of phylogenetic relationships among closely related species and within varieties¹. For example, some taxa of the legume family, complete chloroplast genomes have been identified, in-

cluding 115 genes consisting of 79 protein-coding genes (68.7%), 31 tRNA genes (26.96%), and 5 rRNA (4.35%) [6]. Chloroplast genomes are potential sources of genetic markers for phylogenetic studies, genetic diversity, and molecular identification. [7]. Molecular research methods are widely used to study representatives of the Fabaceae family, among them, there are: *Glycine max* [L.] Merr., *V. radiata* var. *radiata*, *V. angularis* var. *angularis*, *Phaseolus vulgaris* L., *Cicer arietinum*, *Arachis hypogaea*, *Astragalus membranaceus* var. *mongholicus*, *Hedysarum polybotrys* [8–11].

As for the study of the genus *Hedysarum* L., the works by Chennaoui H., Marghali S., Marrakchi M., Trifi-Farah N.

¹ Lu-qi H. Molecular Pharmacognosy. Second Ed.- Springer Nature Singapore Pte Ltd. & Shanghai Scientific & Technic Publish. 2019: 303 p. DOI: 10.1007/978-981-32-9034-1.

should be notified. The phylogenetic relationships within the genus *Hedysarum* L. have been studied on the basis of its morphological and biochemical characters² [12–14]. Nafisi H., Ranjbar M., Wojciechowski M. et al. have carried out molecular genetic studies based on a comparative analysis of ribosomal genes of the genus *Hedysarum* L. species growing in Southeast Asia [15, 16].

The *H. chaiyrakanicum* and *H. theinum* species, for which the authors notified a pronounced polymorphism of the internal transcribed ITS spacers of the 5.8S rRNA gene and the traces of a phylogenetic relationship with Mediterranean species of the genus, have been studied. A relationship is notified between the Asian and European species *H. chaiyrakanicum* and *H. gmelinii*, which belong to the related *Subacaulia* and *Multicaulia* sections [17–19].

In addition to well-known foreign authors, a significant contribution to the genus *Hedysarum* L. was made by domestic authors, including I.A. Shantser and Suprun N.A., who had studied the genetic variation of *H. grandiflorum* Pall., *H. biebersteinii* and *H. argyrophyllum* [4, 20–23]. To study the genetic polymorphism of *Hedysarum* L., the analysis of ISSR markers had been used, which made it possible to analyze more than 100 DNA fragments [24–27].

THE AIM of the research is to study the possibility of using molecular genetic research methods in carrying out complex pharmacognostic studies, to study the intra- and interpopulation variability of three species of the genus *Hedysarum* L. collected in the North Caucasus (*Hedysarum caucasicum* Bieb.(H₁), *Hedysarum grandiflorum* Pall. (H₂), *Hedysarum daghestanicum* Rupr. ex Boiss.(H₃)), to determine possible phylogenetic relationships between the species of the genus *Hedysarum* L.

MATERIALS AND METHODS

The material for the study was the samples of the genus *Hedysarum* L. species gathered in the territory of the North Caucasus: *H. caucasicum* Bieb., collected in the fruiting phase in 2017 in the Alibek gorge, the Dombai section in the territory of the KChR; *H. grandiflorum* Pall., collected in the fruiting phase in 2018 in the village of Kondrashi, the Ilovinsky district of the Volgograd region; *H. daghestanicum* Rupr. ex Boiss, collected in the flowering phase in 2015 in the village of Andi in the Republic of Dagestan [28]. In this work, sequencing was performed according to the Sanger method determining the sequence of ITS1-5.8S-ITS2 rRNA [29]. The DNA sequencing was performed on an AbiPrism 3130 genetic analyzer (Applied Biosystems, USA) at the Biosystematics and Cytology Laboratory in Komarov Botanical Institute of the Russian Academy of Sciences.

The DNA sequence analysis was performed using the MEGA 10.0 software, USA. The isolation of genomic DNA was carried out by the CTAB method from the leaves of the herbarium samples [14]. For the amplification, Dream Taq PCR Master Mix reagents (Thermo Scientific, USA) were used. The polymerase chain reaction was carried out on a C1000 Thermal Cycler (Bio-Rad, USA). The amplification cycle parameters were: 3 min 98°C; 35 cycles: (1 min 98°C; 30 seconds 54°C; 30 seconds 72°C); 10 min 72°C.

The Big Dye Terminator Kit v. 2.0 (Perkin Elmer Life Sciences, Inc., USA) and the ABI Prizm 3130 sequencer (Applied Biosystems, UK) were used for sequencing. The DNA isolation from the leaves or herbarium samples was performed using the CTAB technique and included the following stages: the leaves of the studied samples were ground into a fine powder for 10 seconds using a Tissue-Lyser homogenizer (QIAGEN, USA). Then 700 µl of pre-warmed extraction buffer EB was added and vigorously shaken; it was incubated at 65°C for 1, 2 or more hours; purified with an equal volume of a chloroform mixture: isoamyl alcohol (24:1) was shaken for 5 minutes, the samples were centrifuged for 10 minutes at room temperature at 14000 rpm. The upper phase was transferred into a new 1.5 ml tube, the DNA was precipitated with 2/3 of the isopropanol volume (5 min at room temperature), centrifuged at room temperature for 10 minutes at 14,000 rpm; the supernatant was removed and the supernatant was washed twice with Wash Buffer (WB). The precipitate was air dried and dissolved in 300 µl of TE buffer; 3 µl of RNase L (10 mg/ml) was added and incubated for 30 min at 37°C. The concentration was adjusted with 2M sodium chloride solution; precipitated again by adding 2 volumes of 96% ethyl alcohol, washed with 500 µl of 70% ethyl alcohol, then the granules were air dried and dissolved in TE buffer. For a direct PCR analysis, the Phire Plant Direct PCR Master Mix (Thermo Scientific, USA) was used, which was directly intended for plant leaves and seeds without any preliminary DNA purification.

The amplified DNA fragments were purified using a standard agarose electrophoresis method. The control was carried out visually using a UV transilluminator, since the bands of the DNA stained with fluorescent dyes, formed by molecules of the same size when moving through the pores of the gel, are visible in the UV light. Ethidium bromide ($\lambda_{\text{max}} = 590 \text{ nm}$) which intercalates into DNA molecules (embedded between adjacent pairs of nucleotides), was used as a DNA dye. The intensity of this fluorescence is 20 times higher. The gel strip containing the necessary DNA fragment, was excised. To isolate DNA from the gel, a QIAquick Gel Extraction Kit (QIAGEN, USA) was used.

The ITS1-5.8S-ITS2 marker region of the 5.8S rRNA gene was sequenced in the representatives of the genus *Hedysarum*. The phylogenetic reconstruction was based on a comparison of this marker region from the sequenced samples of different geographic origins and the data from the Genbank NCBI³. To construct phylogenetic trees, the maximum likelihood method was used in the MEGA 10.0 program.

RESULTS AND DISCUSSION

A comparative molecular study of *Hedysarum caucasicum* M. Bieb., *Hedysarum daghestanicum* Rupr. ex Boiss, *Hedysarum grandiflorum* Pall. samples represented in the flora of the Caucasus, were carried out. The ITS1-5.8S-ITS2 sequences of the 5.8S rRNA gene in *Hedysarum caucasicum* were compared with the data presented in Genbank⁴ [30–34]. The resulting phylogenetic tree is shown in Fig. 2.

² Bojnanský V, Fargašová A. Atlas of Seeds and Fruits of Central and East-European Flora: The Carpathian Mountains Region. Dordrecht: Springer. 2007: 1046 p.

³ GenBank Overview. Available from: <http://www.ncbi.nlm.nih.gov/Genbank>.

⁴ Ibid.

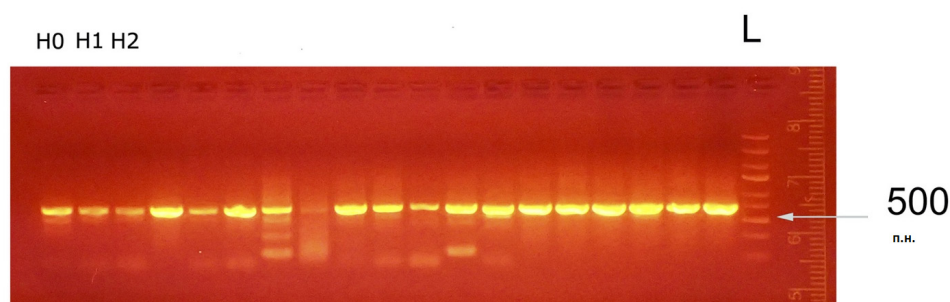


Figure 1 – Results of DNA staining in UV light (ethidium bromide dye)

Note: H₀ – *Hedysarum daghestanicum* Rupr. ex. Boiss; H₁ – *Hedysarum caucasicum* M. Bieb.; H₂ – *Hedysarum grandiflorum* Pall.

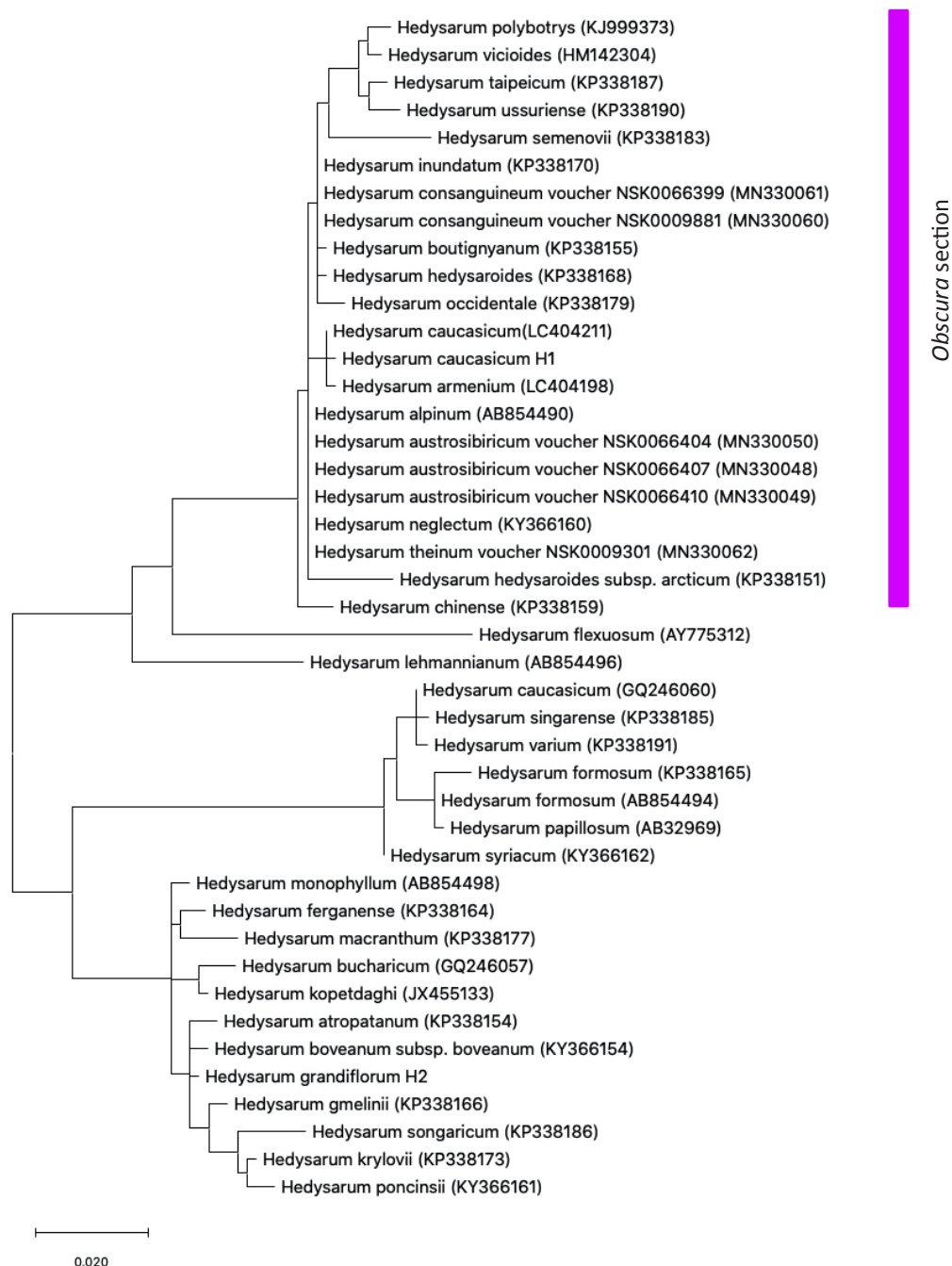


Figure 2 – Phylogenetic relationships of the genus *Hedysarum* L. species

Table 1 – Comparative characteristics of morphological and ecological-phytogenetic features of the genus *Hedysarum* L. species based on the phylogenetic tree growing in the territory of the Russian Federation

SeqNo	Species name	Life form, leaves	Inflorescence elements, flower structure	Phytocenotic type
Section 2. <i>Spinosissima</i>				
1	<i>Hedysarum flexuosum</i> L. ^{5,6}	An annual plant; the stem is strong, brachiate, 50–120 cm high with 2–5-paired leaves, the stipules are large, free; the leaflets are 2–5-paired, almost round or oblong-ovate, scatteredly fluffy below, 15–22 mm long.	The flower spikes are shortly appressed hairy; the inflorescences are elongated; the corolla is purple or pink, 10–12 mm long, the ovary is hairy	Slopes, meadows, deep clay soils; up to 100 m.
Section 3. <i>Obscura</i>				
2	<i>Hedysarum arcticum</i> B.Fedtsch. ⁷	A perennial plant; the stem is strong, glabrous, 20–35 cm high; the stipules are a lot, fused together; the leaflets are 4–9 in number, paired, oblong-elliptical, glabrous above, pubescent below along veins and edges, or almost glabrous, 12–20 mm long.	The flower spikes are axillary, at the beginning of flowering the racemes are compressed, 4–7 cm long; the flowers are 5–30 in the amount, somewhat drooping, purple, violet-red.	In the arctic and adjacent forest zone in the tundra, on sandy islands, in larch sparse forests, in riverside shrubs; in the alpine zone.
3	<i>Hedysarum inundatum</i> Turez. ^{8,9,10}	A perennial plant; the stems are upright, glabrous, 20–40 cm high; the leaves are compound, the leaflets are 5–9 paired, oblong-elliptical, the peduncles are light-brown, 8–12 cm long.	The flower spikes are apical; the racemes are 4–6 cm long; the flowers are 24–35 in the amount, slightly drooping; the floral bracts are light brown, linear-lanceolate, the corolla is 16–20 mm long, mauve.	Along gravels and cliffs in the subalpine and alpine zones, in the zones of mountain ranges.
4	<i>Hedysarum consanguineum</i> DC. ^{11,12}	A perennial plant; the stems are upright, short fluffy or almost glabrous, 20–45 cm high; the leaves are green, forming 2–4 internodes; the stipules are numerous, accrete, short, brown; the leaflets are 4–8-paired, almost glabrous or appressed short hairy.	The flower spikes are apical; the racemes are 10–18 cm long; the flowers are 27–30 in the amount, horizontal or drooping; the calyx is short-campanulate, fluffy, the teeth are lanceolate-subulate; the corolla is 17–19 mm long, mauve.	Through river valleys and swampy forests. In the alpine zone in alpine, subalpine meadows, stony placers, in the tundra.
5	<i>Hedysarum austrosibiricum</i> B.Fedtsch. ^{13,14}	A perennial plant; the stems are upright, strong, glabrous, 20–40 cm high, the stipules are accreted at the base of the stems; the leaflets are 4–9 pairs in number, oblong-elliptical, glabrous above, barely pubescent below along the midrib and edges, 15–25 mm long, 7–10 mm wide.	The flowers are lilac, violet-lilac, 15–30 flowers in dense racemes, elongating with fruits up to 10–15 cm; the bracts are lanceolate, yellow-brown, almost reaching the teeth of the calyx.	In the alpine zone on forest, alpine and subalpine meadows, in the tundra, on rubble and lichen-aceous barrens.

⁵ Fedchenko BA. Flora of the USSR. Family *Leguminosae*. M.-L.: Publishing house of the Academy of Sciences of the USSR. 1948; 3:259–319. Russian
⁶ Castroviejo S, et al. Flora Iberica. Plantas vasculares de la Península Ibérica e Islas Baleares / *Leguminosae* VII(II) (partim). Madrid: Real Jardín Botánico. 2000: 1121 p.
⁷ Fedchenko BA. Flora of the USSR. Family *Leguminosae*. M.-L.: Publishing house of the Academy of Sciences of the USSR
⁸ Ibid.
⁹ Stepanov NV. Vascular Plants of the Yenisei Sayan Mountains. Krasnoyarsk: Siberian Federal University 2016: 593 p. Russian
¹⁰ Flora of Siberia *Fabaceae* (*Leguminosae*). Ed. Polozhiya A.V., Malysheva L.I. Novosibirsk: Science. 1994;9: 280 p. Russian
¹¹ Ibid.
¹² Fedchenko BA. Flora of the USSR. Family *Leguminosae*, 1948. Russian
¹³ Ibid.
¹⁴ Flora of Siberia *Fabaceae* (*Leguminosae*), 1994. Russian

SeqNo	Species name	Life form, leaves	Inflorescence elements, flower structure	Phytocenotic type
6	<i>Hedysarum hedsarioides</i> L. ¹⁵	A perennial plant; the stems are upright, glabrous, 30–60 cm high; the stipules are brown, the lower ones are almost completely fused together, the upper ones are partially fused, with lanceolate-elongated free ends; the leaflets are 6–9 paired, with sparse hairs 12–20 mm long.	The flower spikes are longer than the leaves; the racemes are thin, elongated; the flowers are numerous, 30–40, drooping, dark purple; the calyx is with an oblique limb, the teeth are lanceolate or subulate.	Along mountain meadows in the upper parts of the forest zone.
7	<i>Hedysarum neglectum</i> Ldb. ^{16,17,18}	A perennial plant; the stems are upright, glabrous or short fluffy 25–60 cm high, the stipules are numerous, short, brown, accrete; the leaves are petiolate, the leaves are 4–10-paired, oblong-elliptical, appressedly pubescent on both sides, 17–22 mm long.	The flower spikes are apical, the racemes are loose, the floral bracts are brown, linear-lanceolate, glabrous or slightly pubescent; calyx is short-campanulate, short-fluffy; the corolla is lilac, purple-lilac.	In the alpine zone, in descends into the forests; on subalpine, alpine, forest meadows, rocky slopes, in sparse larch forests.
8	<i>Hedysarum caucasicum</i> M. Bieb. ^{19,20}	A perennial plant; the stems are upright, foliate, 30–60 cm long, the leaflets are 7–12-paired, elliptical or ovate-oblong, with a pointed apex, 12–16 mm long [13].	The flower spikes are longer than the leaves; the racemes have long stems, 1.5–2 times longer than the leaves, not very dense; the corolla is dark purple or crimson [13].	Alpine meadows 1500–3500 m above the sea level, on subalpine and alpine meadows, on moraines, on slide rocks, in crooked forests, on rock ledges.
9	<i>Hedysarum Semenovii</i> Rgl. et Herd. ^{21,22,23}	A perennial plant; the stems are solitary, rarely 2–3 in number, upright, grooved, glabrous or sparsely hairy, 50–120 cm high; the stipules are large, fused to each other, brown, the lowest ones are leafless, amplexicaul; the leaves are shortly petiolate; their petioles, like the leaf axis, are appressed-hairy; the leaves are 4–8-paired, round or orbicular-ovate, tender, glabrous above, scatteredly appressed-hairy below, 15–35 mm long.	The flower spikes are axile, accumbent-hairy, the racemes are oblong, multi-flowered, dense; the floral bracts are lanceolate, brown, 4–6 mm long; the calyx is wide campanulate-shaped, 5–6 mm long, slightly oblique, glabrous or slightly hairy; the corolla is yellow.	On grassy slopes, in the spruce forest belt.
10	<i>Hedysarum alpinum</i> L. ^{24,25,26}	A perennial plant; the stems are upright, glabrous or pubescent in the upper part with short and slightly curly hairs, 40–120 cm high; the leaves are compound, odd-pinnate; the leaflets are 6–11-paired, oblong-ovate, 15–30 mm long.	The inflorescences are long, the racemes are dense; the flowers are in the amount of 20–30, pink, mauve, purple when dried, 5–15 cm long in the racemes; the calyx is 3.5–4.5 mm long, covered with short, slightly curly hairs; the corolla is 13–15 mm long, the ovary is glabrous or pubescent.	On forest meadows, in birch and pine forests, on slopes and on sea cliffs.

¹⁵ Fedchenko BA. Flora of the USSR. Family Leguminosae, 1948. Russian¹⁶ Ibid.¹⁷ Wu ZY, Raven PH, Hong DY. Flora of China (*Fabaceae*). Beijing: Science Press. 2010; 10: 577 p.¹⁸ Flora of Siberia (*Fabaceae* (*Leguminosae*), 1994. Russian¹⁹ Ibid.²⁰ Grossheim AA. Flora of the Caucasus. M.L.: Publishing house of the Academy of Sciences of the USSR. 1952;5: 454 p. Russian²¹ Fedchenko BA. Flora of the USSR. Family Leguminosae, 1948. Russian²² Stepanov NV. Vascular Plants of the Yenisei Sayan Mountains, 2016. Russian²³ Wu ZY, Raven PH, Hong DY. Flora of China (*Fabaceae*), 2010.²⁴ Ibid.²⁵ Fedchenko BA. Flora of the USSR. Family Leguminosae, 1948. Russian²⁶ Flora of Siberia (*Fabaceae* (*Leguminosae*), 1994. Russian

SeqNo	Species name	Life form, leaves	Inflorescence elements, flower structure	Phytocenotic type
11	<i>Hedysarum vicoides</i> Turez. ^{27,28}	A perennial plant; the stem is glabrous, 50–120 cm high; the stipules are accreted in the form of a vagina; the leaflets are 8–9-paired, linear-oblong, glabrous above, scarcely pubescent along the mid-rib and edges below.	The racemes are loose, 15–20 cm long; the flowers are in the amount of 10–30; the calyx is glabrous; the corolla is pale yellow, 10–12 mm long.	In river valleys, on the meadows, on riverine gravels, in larch and pine forests.
12	<i>Hedysarum ussuriense</i> I. Schischk&-Kom. ^{29,30}	A perennial plant with a thick, woody, with a long tap root; the stems are numerous, up to 50 cm high; the leaves are odd-pinnate; the leaflets are 3–5-paired, oblong-ovate, almost glabrous, 10–20 mm long	The flowers are whitish-yellow, numerous (up to 20), in a loose, unilateral, racemose inflorescence, on a long flower spike exceeding the leaves.	On limestone rocks and open rocky slopes up to 700 m above the sea level. In some places it forms thickets. Obligate calciphile.
13	<i>Hedysarum theinum</i> Krasnob. ^{31,32}	A perennial herb; the stems are grooved, glabrous or slightly appressed-hairy, 80 cm high, the stipules are brown, fused together, large, lepidodendroid; the leaflets are 2–5-paired, short-petiolate, elliptical or ovate-elliptical, glabrous above.	Purple-lilac flowers are organized in loose racemes of 15–30 pcs.	Hedysarum L. grows on alpine and subalpine meadows, on grassy and rocky slopes, in calciphile forests and on river gravels.
14	<i>Hedysarum armenium</i> Boiss. et Tchih. ³³	A perennial herb; the stems are upright, shortened, 10–30 cm high, the stipules are light brown, wide; the leaflets are 10–13-paired, elliptical or oblong-elliptical, dark green, 8–12 mm long [13].	The racemes are dense, 20–30 flowers in each; the corolla is 15–16 mm long, dark purple, the ovary is pubescent [13].	On mountain meadows, at an altitude of 2100–3000 m [13].
Section 4. <i>Multicaulia</i>				
15	<i>Hedysarum formosum</i> Fisch. et Mey. ^{34,35}	A perennial herb; the stems are coriaceous, up to 5 mm in diameter, upright, slightly flexuose, brachiate, 40–70 cm high; the stipules are large, lanceolate, the lower ones are fused, the upper ones are usually free; the leaflets are 6–10 paired, elliptical, glabrous above, pubescent below [13].	The racemes are elongated; the calyx teeth are subulate from the lanceolate base; the corolla is yellow, 13–15 mm long, pale [13].	Solonized steppes [13].
16	<i>Hedysarum atropatanum</i> Bge. ex Boiss. ^{36,37}	A perennial herb; the stems are white, sparsely silvery-hairy, 30–40 cm high; the stipules are white-membranous, appressed-hairy, short-triangular; the leaflets are 6–10 paired, rounded-elliptical or oblong, smothered with appressed silvery hairs on both sides, 10–12 mm long [13].	The racemes are elongated; 10–15 flowers in each; the calyx is campanulate-shaped, its teeth are subulate from the narrow-lanceolate base, glabrous upwards; the corolla is pink-violet, 15–18 mm long [13].	On clay-slates [13].

²⁷ Ibid.²⁸ Fedchenko BA. Flora of the USSR. Family *Leguminosae*, 1948. Russian²⁹ Ibid.³⁰ Stepanov NV. Vascular Plants of the Yenisei Sayan Mountains, 2016. Russian³¹ Flora of Siberia *Fabaceae (Leguminosae)*, 1994. Russian³² Fedchenko BA. Flora of the USSR. Family *Leguminosae*, 1948. Russian³³ Ibid.³⁴ Ibid.³⁵ Grossheim AA. Flora of the Caucasus, 1952. Russian³⁶ Ibid.³⁷ Fedchenko BA. Flora of the USSR. Family *Leguminosae*, 1948. Russian

SeqNo	Species name	Life form, leaves	Inflorescence elements, flower structure	Phytocenotic type
17	<i>Hedysarum kopetdaghi</i> Boriss. ³⁸	A perennial multicaulis herb; 25–35 cm high, the stipules are membranous, 5–7-paired leaflets, which are oblong-elliptical, obtuse at the apex, with a short mucro, rounded-wedge-shaped to the base, appressed hairy on both sides, 15–30 mm long.	The inflorescences are racemes; the flowers have long stems; the floral bracts are filiform, membranous, up to 5 mm long, the calyx is wide campanulate-shaped, the corolla is purple.	On grassy mountain slopes, at an altitude of 1800–2000 m.
18	<i>Hedysarum varium</i> Willd. ^{39,40}	A perennial plant; the stems are ascending, numerous, brachiate, 20–40 cm high, the stipules are accrete, appressed-hairy; the leaflets are 3–5-paired, oblong or elliptical, glabrous above, fluffy below [13].	The racemes are dense, equal to or longer than the leaves; the corolla yellow; its purple carina is at the apex [13].	On dry slopes [13].
19	<i>Hedysarum Gmelinii</i> Ldb. ^{41,42}	A perennial plant; the stems are few, almost upright, pubescent, with appressed hairs, below 2–3 mm in diameter, the stipules are membranous, accrete, the leaflets are 5–11-paired, oblong, hairy below, sometimes glabrous above, 7–30 mm long.	The flower spike is longer than leaves; the racemes, 15–30 in number, are flowering, dense, elongated at the end; the floral bracts are lanceolate, the calyx is appressed-hairy; the corolla is pink-purple.	On meadows and in stony steppes.
20	<i>Hedysarum songaricum</i> Bong. ^{43,44}	A perennial plant; the stems are well developed, numerous, almost glabrous, 25–60 cm high; the stipules are membranous, the lower ones are fused at the base, slightly colored, hairy; the leaflets are 5–8 paired, lanceolate or oblong-elliptical, glabrous above, about 20 mm long.	The racemes are multi-flowered, oblong; the floral bracts are scarious, lanceolate; the calyx is campanulate-shaped, with lanceolate-subulate teeth, the corolla is pink-violet.	In steppes, on rank soil and finely earthy slopes in the middle and lower mountain belts ^{17,20}
21	<i>Hedysarum atropatanum</i> Bge. ex Boiss. ⁴⁵	A perennial plant; the stems are brachiate, silvery hairy; the stipules are white-membranous, appressed-hairy; the leaves short-petiolized; leaflets 4–6-paired, rounded-elliptical or oblong, 10–12 mm long.	The racemes are elongated, 10–15 flowers in each; the calyx is campanulate-shaped, the corolla is pink-violet, 14–15 mm long.	On clay-slates.
Section 5. Subacaulia				
22	<i>Hedysarum Lehmannianum</i> Bge. ⁴⁶	A perennial acaulous plant; the stems are appressed hairy, 15–35 cm high; the stipules are oblong-lanceolate, brown, fused to each other, appressed-hairy; the leaflets are 7–12-paired, oblong-elliptic, smoothly appressed-hairy below, pubescent above, 10–18 mm long.	The racemes are pretty compressed, multi-flowered; the flowers are 12–20 in the amount, the calyx is divaricately-hairy, the teeth are lanceolate-subulate; the corolla is purple.	On rocky mountain slopes, through bushes in subalpine steppes
23	<i>Hedysarum monophyllum</i> Boriss. ⁴⁷	A perennial sericeous hairy plant 5–12 cm high; the stipules are imbricate at the base of leaves, the stipules are sericeous with strigose; the leaflets are simple, bottom sand, with a rounded-ovoid plate, sericeous with strigose, rounded or acute leaf apexes, sometimes sinuated, rounded at the base, 10–40 mm long.	The inflorescence is rounded-cephalanthium, compact, the floral bracts are ovoid, white-hairy outside, the calyx is wide campanulate-shaped, the corolla is dry, yellowish.	On stony and clay slopes of mountains, at an altitude of up to 2500 m.

³⁸ Ibid.³⁹ Ibid.⁴⁰ Grossheim AA. Flora of the Caucasus, 1952. Russian⁴¹ Flora of Siberia *Fabaceae (Leguminosae)*, 1994. Russian⁴² Fedchenko BA. Flora of the USSR. Family *Leguminosae*, 1948. Russian⁴³ Ibid.⁴⁴ Wu ZY, Raven PH, Hong DY. Flora of China (*Fabaceae*), 2010.⁴⁵ Fedchenko BA. Flora of the USSR. Family *Leguminosae*, 1948. Russian⁴⁶ Ibid.⁴⁷ Ibid.

SeqNo	Species name	Life form, leaves	Inflorescence elements, flower structure	Phytocenotic type
24	<i>Hedysarum grandiflorum</i> Pall. ⁴⁸	A perennial acaulous plant with a tigellum (rarer); peже стебли сильно укороченные, the stipules are large, leath-erly, membranous, accrete, brown, sparsely hairy; the leaflets are 1–4 paired, ovate or wide elliptic, smoothly sil-ver-sericeous beneath, 20–30 mm long [13].	The racemes are multi-flowered, with deviated flow-ers; the floral bracts are lanceolate, light brown, hairy, the calyx is campanulate-shaped; the corolla is yellow or purple-violet [13].	Forest-steppe, stony and thyme steppes, rubble and clay-limestone slopes, limestones, chalk steppes, on outcrops of chalk and marl [13].
25	<i>Hedysarum ferganense</i> Korsh. ^{49,50}	A perennial acaulous plant; 10–30 cm high, the leaves are 4–7-paired, oblong or elliptical, covered with short white, appressed hairs, on the upper surface of the leaves the pu-bescence is not so dense, 10–18 mm long.	The floral bracts are brownish, lanceolate; the raceme is dense; the calyx is campanulate-shaped, its teeth are linear, 2–3 times longer than the tube, covered with adpressed or somewhat divaricately hairs; the corolla is lilac-violet.	On mountain meadows, on stony and gravelly slopes.
26	<i>Hedysarum Poncinsii</i> B. Franchet. ⁵¹	A perennial acaulous plant; densely white-tomentose, 5–10 cm high, the stipules are white-membranous, with a slight admixture of brownish color; the leaflets are 3–4-paired, oblong-ovovate, 5–7 mm long.	The raceme is compressed, cephalanthium; the calyx is campanulate-shaped, its teeth are linear-filiform, covered with detached hairs; the corolla is dark pur-ple.	Steppes, at an altitude of 2800–3200 m.
27	<i>Hedysarum Krylovii</i> Sumn. ^{52,53}	A perennial plant; the stipules are white, scarious, ap-pressed hairy; the leaflets are 3–7-paired, oblong-lanceo-late, glabrous or with slightly appressed hairs, silvery be-neath, 10–23 mm long.	The inflorescence is multi-flowered, oblong, less often ovate, the floral bracts are nearly equal to the calyx tube; the flowers are purple.	In solonized steppes and gravelly terraces.
28	<i>Hedysarum dagh-estanicum</i> Rupr. ex Boiss. ^{54,55,56,57}	A perennial plant; gray in color because of appressed pubescence; the stipules accreted; the leaves are covered with sericeous pubescence on both sides; the leaflets are 2–3-paired, oblong or ovate-lanceolate, acute, the apical leaflet is larger, up to 18 mm long.	The raceme is pauciflorous, dense; the flowers are big, creamy-white or purple [13].	Calcareous and dry grassy slopes, rocky places, at an altitude of from 800 to 1500 m [13].
Section 6. <i>Grinifera</i>				
29	<i>Hedysarum macranthum</i> Freyn. et Sint. ⁵⁸	A stemless or almost stemless perennial; 20–30 cm high; the stipules are sericeous, fused together, lanceolate; the leaves are 5–7 paired.	The racemes are longer than leaves, with acute apices; the floral bracts are hairy; the calyx is wide campanulate.	On stony and gravelly slopes.
30	<i>Hedysarum bucharicum</i> B. Fedtsch. ⁵⁹	A perennial plant; the stems are thin, ascending, ap-pressed-hairy; the stipules are pale, fused at the bases, tri-angular-lanceolate; the leaflets are 5–8-paired, oblong-lan-ceolate, 14–18 mm long.	The racemes are not dense; the flowers are 12–25 in the amount; the floral bracts are nondeciduous; the corolla is bright purple.	Gypsum and limestone, among juniper woodlands and absinthial mixed herbs communities.

⁴⁸ Ibid.⁴⁹ Ibid.⁵⁰ Wu ZY, Raven PH, Hong DY. Flora of China (*Fabaceae*), 2010.⁵¹ Stepanov NV. Vascular Plants of the Yenisei Sayan Mountains, 2016. Russian⁵² Ibid.⁵³ Flora of Siberia (*Fabaceae* (*Leguminosae*), 1994. Russian⁵⁴ Stepanov NV. Vascular Plants of the Yenisei Sayan Mountains, 2016. Russian⁵⁵ Litvinskaya SA, Murtazaliev RA. Flora of the North Caucasus: Key Atlas. Moscow: Fiton XXI. 2013: 688 p. Russian⁵⁶ Grossheim AA. Flora of the Caucasus, 1952. Russian⁵⁷ Galushko AI. Flora of the North Caucasus: key in 3 vol. Publishing house of Rostov University, 1980; 2: 352 p. Russian⁵⁸ Stepanov NV. Vascular Plants of the Yenisei Sayan Mountains, 2016. Russian⁵⁹ Ibid.

Table 2 – Chromosome numbers of the genus *Hedysarum* L. species

Seq No.	Species name ^{60,61,62,63}	GenBank number ⁶⁴	Chromosomal complement
Section 1. <i>Fruticosa</i>			
1.	<i>Hedysarum fruticosum</i> Pall. [13]	–	2n=16
Section 2. <i>Spinosissima</i>			
2.	<i>Hedysarum flexuosum</i> L.	AY775312	2n=16
Section 3. <i>Obscura</i>			
3.	<i>Hedysarum arcticum</i> B. Fedtsch.	KP338151	2n=14
4.	<i>Hedysarum inundatum</i> Turez.	KP338170	2n=8, 2n=28
5.	<i>Hedysarum consanguineum</i> DC.	NSK0066399 (MN330061); NSK0009881 (MN330060)	2n=14
6.	<i>Hedysarum austrosibiricum</i> B. Fedtsch.	NSK0066407 (MN330048); NSK0066410 (MN330049); NSK0066404 (MN330050)	2n=14
7.	<i>Hedysarum hedysaroides</i> L. [16]	KP338168	2n=14
8.	<i>Hedysarum neglectum</i> Ldb.	KY366160	2n=14
9.	<i>Hedysarum caucasicum</i> M. Bieb.	GQ246060; LC404211; RC – The object under study	2n=14
10.	<i>Hedysarum Semenovii</i> Rgl. et Herd.	KP338183	–
11.	<i>Hedysarum alpinum</i> L.	AB854490	2n=14
12.	<i>Hedysarum vicoides</i> Turez.	HM142304	2n=14
13.	<i>Hedysarum ussuriense</i> I. Schischk & Kom.	KP338190	2n=16
14.	<i>Hedysarum theinum</i> Krasnob.	NSK0009301 (MN330062)	–
Section 4. <i>Multicaulia</i>			
15.	<i>Hedysarum formosum</i> Fisch. et Mey.	AB854494; KP338165	–
16.	<i>Hedysarum atropatanum</i> Bge. ex Boiss.	KP338154	–
17.	<i>Hedysarum kopetdaghi</i> Boriss.	JX455133	–
18.	<i>Hedysarum varium</i> Willd.	KP338191	–
19.	<i>Hedysarum Gmelinii</i> Ldb. [15]	KP338166	2n=28
20.	<i>Hedysarum songaricum</i> Bong.	KP338186	–
Section 5. <i>Subacaulia</i>			
21.	<i>Hedysarum Lehmannianum</i> Bge.	AB854496	–
22.	<i>Hedysarum monophyllum</i> Boriss.	AB854498	–
23.	<i>Hedysarum grandiflorum</i> Pall.	The object under study	–
24.	<i>Hedysarum ferganense</i> Korsh.	KP338164	–
25.	<i>Hedysarum setigerum</i> Turcz.	–	2n=28, 32, 48
26.	<i>Hedysarum Poncinsii</i> B. Franchet.	KY366161	–
27.	<i>Hedysarum Krylovii</i> Sumn.	KP338173	–
Section 6. <i>Crinifera</i>			
28.	<i>Hedysarum macranthum</i> Freyn. Et Sint.	KP338177	2n=16
29.	<i>Hedysarum bucharicum</i> B. Fedtsch.	GQ246057	–

Table 3 – The content of xanthenes in various species of the genus *Hedysarum* L.

Seq No.	Species name	The sum of the xanthenes
Section 3. <i>Obscura</i>		
1.	<i>Hedysarum austrosibiricum</i> B. Fedtsch.	3.0
2.	<i>Hedysarum neglectum</i> Ldb.	3.6
3.	<i>Hedysarum flavescens</i> Rgl. et Schmalh.	5.5
4.	<i>Hedysarum caucasicum</i> M. Bieb.	4.0
5.	<i>Hedysarum alpinum</i> L.	6.0
6.	<i>Hedysarum theinum</i> Krasnob.	3.1
Section 4. <i>Multicaulia</i>		
7.	<i>Hedysarum Gmelinii</i> Ldb.	1.2

Some of the studied species form well-maintained clades. That can be said about the species assigned to the *Obscura* section. As a result of the research, a comprehensive ecomorphology and molecular genetic analysis has been carried out, which makes it possible to carry out a correlation between morphological, ecological-phyto-

cenotic, molecular-genetic indicators. 29 species of 309 species of the genus *Hedysarum* L., which belongs to the Fabaceae family, grow in the territory of the Russian Federation, 17 species are found in the flora of the Caucasus.

The genus *Hedysarum* L. includes 6 sections: *Fruticosa*, *Spinosissima*, *Obscura*, *Multicaulia*, *Subacaulia*,

⁶⁰ Stepanov NV. Vascular Plants of the Yenisei Sayan Mountains, 2016. Russian

⁶¹ Flora of Siberia *Fabaceae* (*Leguminosae*), 1994. Russian

⁶² Bolkhovskikh ZV, Grif VG, Zakhariyeva OI, Matveeva TS. Chromosome numbers of flowering plants. L.: Nauka. 1969 : 927 p. Russian

⁶³ Castroviejo S, et al. Flora Iberica. Plantas vasculares de la Península Ibérica e Islas Baleares / *Leguminosae* VII(II) (partim), 2000.

⁶⁴ GenBank Overview. Available from: <http://www.ncbi.nlm.nih.gov/Genbank>.

Crinifera. Such morphological indicators as a life form, peculiarities of the leaves structure, elements of the generative organs structure (flowers and fruits) are depicted. In addition, for each species, ecological and phytocenotic characteristics have been indicated, including the peculiarities of growth and altitude level (Table 1).

When analyzing the data obtained, it should be notified the assumed correlation between the habitat of alpine species living mainly on rocks, gravels, rockslides, in the subalpine and alpine belts with moist soil and purple or pink-violet color of the corolla petals. The examples are the species such as: *Hedysarum flexuosum* L., *Hedysarum arcticum* B. Fedtsch., *Hedysarum inundatum* Turez., *Hedysarum austrosibiricum* B. Fedtsch., *Hedysarum hedysaroides* L., *Hedysarum neglectum* Ldb., *Hedysarum caucasicum* M. Bieb., *Hedysarum alpinum* L., *Hedysarum theinum* Krasnob., *Hedysarum kopetdaghi* Boriss., *Hedysarum songaricum* Bong.

Herewith, a group of yellow-flowered *Hedysarum* L. growing mainly on solonized, dry limestone slopes with a low level of soil moisture, on gravels, on clay-slates, can be distinguished. These species can be classified as calciphiles, among them there are: *Hedysarum Semenovii* Rgl. et Herd., *Hedysarum vicioides* Turez., *Hedysarum ussuriense* I. Schischk. & Kom., *Hedysarum formosum* Fisch. et Mey., *Hedysarum varium* Willd., *Hedysarum grandiflorum* Pall. The analysis of chromosome numbers of species of the genus *Hedysarum* has been carried out [35, 36]. The results are shown in Table 2.

The chemical composition of the genus *Hedysarum* L. species has been studied for the accumulation of the main marker group of biologically active substances – xanthone glycoside mangiferin [37–40]. The results are shown in Table 3.

Based on the foregoing, it can be concluded that the species of the section *Obscura* are characterized by a maximum accumulation of xanthone glycosides, mainly mangiferin. Thus, for *Hedysarum alpinum* L. the content was 6.0%; for *Hedysarum flavescens* Rgl. et Schmalh it was 5.5%, and for *Hedysarum theinum* Krasnob. – 3.1%.

Based on the studies carried out, it is possible to suggest several directions of phylogenetic regularities concerning the correlation between the chemical composition of the main groups of biologically active substances involved in the metabolism of this genus, including the marker xanthone glycoside mangiferin, and a change in the color of the corolla petals. A separate direction can be considered a possible correlation between the ecological and phytocenotic characteristics of the growth of species and the accumulation of the main groups of xanthones. As a result of the molecular analysis, it can be assumed that it is possible to make a forecast of additional raw materials sources of mangiferin and other groups of xanthones from the representatives of the *Obscura* section. The analysis of phylogenetic relationships of this section representatives of the genus *Hedysarum* L. species confirms the morphological classification of this genus.

The section *Obscura* which comprises more than 20 species, including the *Hedysarum caucasicum* studied in this article, is characterized by similar morphological and ecological-phytocenotic parameters. According to the GenBank⁶⁵ data, almost all of these species belong to this section, which completely confirms the correlation between the classical taxonomic and molecular genetic methods. This section is characterized by a significant accumulation of mangiferin – from 3.0 to 6.0%. In the aerial part of *Hedysarum caucasicum*, up to 4.0% of the amount of xanthones in terms of mangiferin has been found. It should be notified that this group of species grows mainly in the alpine and subalpine zones and has a characteristic violet-purple color of the corolla petals.

The *Subcaulia* section, which includes 22 species, is characterized by arid habitats, dry rocky limestone slopes. This group is calciphilic, with a predominantly yellow color of the corolla petals. The representatives of this section, including *Hedysarum* L. large-flowered, accumulate a small amount of xanthones – from 0.05 to 0.1%.

When analyzing the data obtained, it should be notified that the observed correlation exemplified by the representatives of the genus *Hedysarum*, makes it possible not only to establish the degree of phylogenetic relationships of various taxa [8, 9, 40–45], but also to represent the totality of chemotaxonomic characteristics of certain species using the example of the *Fabaceae* family. In the future, the data can be used in pharmacognostic studies and the development of regulatory documents for medicinal plant raw materials.

CONCLUSION

Preliminary results have been obtained. This fact makes it possible to carry out a comprehensive identification of the studied samples of the genus *Hedysarum* L. species (*Hedysarum caucasicum* M. Bieb., *Hedysarum daghestanicum* Rupr. ex Boiss., *Hedysarum grandiflorum* Pall.), growing in various high-mountainous areas of the North Caucasus. Preliminary results of the application of molecular genetic research methods in the analysis of the genus *Hedysarum* L. are presented in this article. For the first time, nucleotide substitutions in the 5.8S rRNA gene sequence of the genus *Hedysarum* L. three species have been identified, and the phylogenetic tree of the genus *Hedysarum* L. is presented.

It should be notified that in the future, the observed correlation between the accumulation of secondary metabolites of the xanthone series and the ecological and phytocenotic characteristics, can serve to isolate the species of the *Obscura* section to search for additional raw material sources of mangiferin and its derivatives with a pronounced antiviral activity.

Taking into account the development of modern molecular pharmacognosy, the data obtained can be further used in the preparation of regulatory documents for medicinal plant raw materials.

⁶⁵ Ibid.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

AUTHORS' CONTRIBUTION

Javgarat R. Imachueva – setting up the experiment, discussing the results; Fatima K. Serebryanaya – project management, collection of plant material, discussion of the results; Eduard M. Machs – setting up an experiment, discussing the results; Violetta V. Kotseruba – project management, discussing the results.

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