Comparative Analysis of Anatomical, Phytochemical and Molecular Genetic Characteristics of Two Species of *Hedysarum*l.

A.B. Kusmangazinov
Al-Farabi Kazakh National University
Meruyert Sakenovna Kurmanbayeva (kurmanbayevakz@gmail.com)
Al-Farabi Kazakh National University https://orcid.org/0000-0002-5050-9142
I.M. Zharkova
Al-Farabi Kazakh National University
A. K. Sarkytbayeva
Al-Farabi Kazakh National University
T.K. Kayirbekov
Ondokuz Mayis University: Ondokuz Mayis Universitesi
M. Hohn
Hungarian University of Agriculture and Life Sciences - Kaposvár Campus: Magyar Agrar- es Elettudomanyi Egyetem - Kaposvari Campus

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Abstract

Background

Hedysarum L. is one of the large families of the Fabaceae, consisting of 200 species. Species in the genus Hedysarum are valuable forage and medicinal resources. In this paper we studied *Hedysarum theinum* Krasnob. and *Hedysarum neglectum* Ledeb. growing in the Western Altai of Eastern Kazakhstan. *H.theinum* is a perennial, herbaceous, valuable medicinal plant, a rare high-altitude species, an Altai-Dzungarian endemic and globally endangered species. *H. neglectum* is a perennial plant of high medicinal and fodder value, growing mainly in the sub-alpine zone. A complex phytochemical study of *Hedysarum* using modern methods, expanding the data on herbal medicines is actual. At the current time, taxonomic and phylogenetic questions of the genus *Hedysarum* are the subject of close attention, as the systematic knowledge of the genus *Hedysarum* L. Fabaceae: Hedysareae is still incomplete. Therefore, questions are being actively addressed by correlating data obtained by molecular genetic analyses. In the study, route-recognition methods were used in a field search of two species of *Hedysarum* for comparative morphological study in 2020–2021.

Results

The morphological measurements showed a difference in the external structure of the two species. Structural changes in the anatomical structure of the leaf, stem and root were analyzed as the plants adapted to different conditions in the Western Altai. The changes were expressed in the transition from the typical dorsiventral leaf structure of *H.theinum* to the isolateral-palisade structure of the mesophyll of *H.neglectum.* The phytochemical analysis revealed the highest amount of ethyl α-d glucopyranoside at 45.23% in the subterranean part of *H. theinum*, which was only 0.8% in *H. neglectum*. The root part of *H. theinum* contained the highest vitamin E content of 11.36%, while the above-ground part of *H. neglectum* showed an insignificant amount of tocopherol at 0.37%. Also the above-ground and underground parts of the two species contain squalene, which has an antioxidant effect and strengthens the immune system. Nowadays, an urgent task in pharmacognosy is the use of all kinds of DNA analysis methods for the identification of plant raw materials. In this regard, the possibility of using molecular genetic methods of research in the analysis of representatives of the genus *Hedysarum* L., for the identification of medicinal plant raw materials was studied.

Conclusion

Phylogenetic analysis using molecular genetic markers revealed that *H. neglectum* and *H. theinum* belong to the Hedysarum section. The data obtained can be used for the species identification of *H.theinum* and *H.neglectum*, which has so far been difficult due to the lack of some systematic morphological characters. The results obtained may be useful for the taxonomic studies of this section.

Background

The family Fabaceae consists of about 20 000 species belonging to some 751 genera. *Hedysarum* is the second largest genus in the tribe Hedysareae (Fabaceae). One important species of the genus *Hedysarum* is *Hedysarum theinum*, which grows in Kazakhstan. The species is vegetatively immobile and reproduces only by seeds. *Hedysarum theinum* is resistant to disease and has a fairly high seed production. The introduction assessment indicates a high plasticity and high degree of adaptation of the species (Zinner et al. 2021). However, in the Republic of Altai and Northern Kazakhstan, an average seed production is noted, which is sufficient for regular renewal of populations and maintaining the stability of their age structure (Karnaukhova et al. 2021). Based on the analysis, the relationship between morphological variability of productivity traits and genetic variability of electrophoretic polypeptide spectra of seeds of valuable medicinal species *H. theinum.* (Erst et al. 2014).

The adaptation of the species *H. theinum* by anatomical features of leaf blade structure to high-gradient habitats of South Siberia was traced; it was revealed that heliomorphic features are intensified (Karnaukhova et al. 2018 and Karnaukhova 2016). *H.theinum* are protected and included in the Red Data Book of the Altai Republic as rare or declining species (Artemov, 2018).

Representatives of the genus *Hedysarum* L. are promising medicinal and high-protein fodder plants with high trypsin-inhibiting activity in leaves. Trypsin inhibitory activity is one of the immunity factors in plants that ensures the presence of general non-specific systemic resistance. In leaves of *H. theinum* values of trypsin-inhibitory activity reaches a maximum only in favorable conditions during flowering phase of seasonal development of plants in the Altai Republic (Zhmud et al. 2020).

The study of the antidepressant action of species of the genus *Hedysarum* roots *H. theinum*, roots *H. ignorecum* Ledeb., herbs *H. alpinum* L. showed pronounced anti-anxiety effects of extracts of medicinal plants and the complete absence of depressant effect, which justifies the possibility of using these drugs for the prevention and correction of mental disorders associated with aggression, increased levels of anxiety, cognitive impairment (Fedorova et al.2021). Study of the antidepressant properties of some plants.

The use of one of the valuable medicinal plants, *H. theinum* is of particular interest for public health. *Hedysarum* finds widespread use in folk medicine, hardly used in official medicine due to its practically unexplored phytochemical composition. Although, modernmedicine recognizes that this plant has medicinal properties for many diseases due to the specific combination of valuable biologically active compounds.

H.theinum - red root, of the family Fabaceae Lindl. is a mountainous-Altai-Middle Asian-Mongolian endemic, classified as category 2a as a globally declining species. The plant is perennial, with thickened roots, bare stems, stipules large, fused, leaves elliptic, 4–8 paired, corolla pinkish-purple, seeds ovoid. Western Altai plants are characterized by the following dimensions: stems 45–70 cm high and finer leaflets about 2 cm long and 0.6-1.0 cm wide, while Southern Altai forms differ in size; the stem is almost

twice as high as the Western Altai species and the stem reaches up to 1 cm thick with 11–13 nodes and more 115 large leaves, 4 cm long and 1 cm wide.

Outside Kazakhstan, *H. theinum* is distributed in the Russian part of the Western and Central Altai and in the mountain ranges of western Mongolia. It belongs to the mesopsychrophyte ecological group. Occurs in humid conditions of mountain systems, the altitude of which reaches 1700–2100 m above sea level. It prefers moist, humus-rich soils (Kuminova 1960).

H. theinum is also very similar to *H. neglectum*, with which it is often mixed, thus differing in structure and chemical composition of the root. It has shorter and denser multifarious inflorescences, and is also distinguished by shorter pedicels, longer bracts, bracts reaching the apex of the calyx teeth, filiform teeth of the larger calyx, larger calyx, larger flowers with a pod (Krasnoborov et al. 1985).

According to the research of Polozhiy, *H.theinum* is a relic of the glacial epoch, isolated from the older boreal-forest *H. alpinum*. Within Kazakhstan, it occurs in the Southwestern and Southern Altai ranges, Southwestern Altai: Ivanovsky (Polozhiy 1972).

Of particular importance is the fact that in natural populations *H. theinum* is morphologically similar to the species *H. neglectum*, which is controversial among scientists. Comprehensive morphological, anatomical and genetic studies are needed to clarify this issue, taking into account the natural conditions of the plants. *H. theinum* is poorly studied chemically and ecologically; of particular interest is the unique phytochemical composition capable of treating tumour diseases and requiring in-depth study of this plant (Kupriyanov et al. 2003).

H. neglectum a perennial plant 25–50 cm high with thickened roots, producing erect or slightly ascending, furrowed, sparsely adpressed hairy or glabrous (2–5) stems; stipules large, intergrown, brown; stem leaves 4–6, shortly petiolate, leaflets are 4–8 paired, elliptic or ovate-elliptic, about 20 mm long, 10 mm wide, glabrous above, shortly hairy along edges and lower main vein. Racemes oblong, with drooping flowers; bracteoles lanceolate, up to 10 mm long, lanceolate; calyx bell-shaped, with teeth equal to or slightly longer than tube; corolla pink-purple, flagellum oblong-ovate, 15–16 mm long. Wings oblong-linear, nearly as long as flagellum, with narrow-linear sepals, about 3 mm long, marigold 18–20 mm long, hanging beans, 3-5-segmented, segments rounded, seeds ovoid, 3 mm long, olive-brown. Blossoms in July and August (Flora of Kazakhstan 1956–1966).

The territory of Kazakhstan is a large stock of medicinal plants, which have long been widely used in traditional, folk medicine, but have not found application in official medicine. One such plant is *H. theinum* and *H. neglectum* from the Fabaceae family, which have been used in folk medicine in the Altai for centuries to treat a wide range of human ailments (Sviridova et al. 2011).

The ITS analysis demonstrated the polyphyletic nature of the genus *Hedysarum*, whose members were unevenly distributed among species of other genera in the phylogenetic tree of the tribe Hedysareae. Therefore, an extended molecular phylogenetic study of the tribe Hedysareae has been proposed using

cDNA marking. To study the phylogeny of the genus and to differentiate species, recent nucleotide sequence studies of nuclei and chloroplast origins have confirmed the polyphyletic nature of the genus *Hedysarum* (Nuzhdina et al. 2018).

The high genetic similarity (I = 0.875) found between populations of *H. theinum*, which reflects the highly restricted endemic range of this species, facilitating gene flow between populations and outcrossing (Zviagina et al. 2013). Variability in seed polypeptide profiles in highland *H. theinum* populations was lower than in forest populations (Dorogina et al. 2009). On the contrary, the species *H. neglectum* was found to have changes in leaf structure in foothill plains and in the altitude gradient of habitats; this can be considered as important biological adaptations to extreme growing conditions (Karnaukhova et al. 2018).

Objects And Research Methods

Samples of plants of *H. theinum* and *H.neglectum* of Fabaceae family were collected from natural populations of Kazakhstan Altai in natural habitats in the territory of Western Altai, in the valley of Big Poperechka river, Ivanovo ridge (Fig. 1), during the period from May 2020 to August 2021. In order to study the current state in natural habitats in 2020–2021, itinerary and reconnaissance field surveys were conducted across the Western Altai in Kazakhstan Altai, forming a number of small ridges, depressions, and river valleys. The 15 plant samples were collected for anatomical examination and recorded, 15 samples were collected for phytochemical analysis, dried and crushed, and fresh leaves from 20 individuals of each population were collected for molecular genetic study, Table 1- Presented GPS coordinates of two *Hedysarum* species.

Table 1
Information on the material collected

Name of species	Coordinates [°]		Height above sea level	Gathering point
	Longitude	Latitude		
Hedysarum theinum Krasnob.	50° 20' 10" N	83°53'14" E	1470	East Kazakhstan region, vicinity of Ridder town, Ivanovsky ridge, valley of the Big Poperechka river
Hedysarum theinum Krasnob.	50°19' 59" N	83°52'44" E	1812	East Kazakhstan region, vicinity of Ridder town, Ivanovsky ridge, on a rocky mountainside
Hedysarum neglectum Lindl.	50°18' 50" N	83°52'42" E	1938	East Kazakhstan region, vicinity of Ridder town, Ivanovsky ridge, on a motley grassy mountainside

Studies were conducted using semi-stationary methods (Shcherbakov et al. 2006). Under field conditions, we described the identified populations with *H.theinum* and *H.neglectum*, established floristic composition (Korchagin 1964), abundance according to Drude (Poniatovskaya 1964), collected

herbarium and collected samples of above- and below-ground plants for further anatomical, phytochemical and molecular genetic studies. Latin names were clarified according to Cherepanov (1995), species identification according to Flora of Kazakhstan, Vol. 1–9 (Flora of Kazakhstan 1956–1966).

Raw material samples for microscopic study were freshly fixed in Strasburger-Fleming's solution (96% ethyl alcohol : glycerol : water in a 1:1:1 ratio) (Prozina 1960; Dolgova et al. 1977). Temporary preparations were prepared from transverse sections, measured and followed by microphotography with an inverted microscope MX 700, Austria.

Statistical processing of research materials was performed according to the method of G.F. Lakin (Lakin 1990). Phytochemical analysis was performed by gas chromatography with mass spectrometric detection (Agilent 7890A/5975C). Quantification of organic substances was carried out by hydrodistillation followed by volume weight analysis of the product (Muzychkina et al. 2011; Litvinenko 2012), also using EXCEL-2010 statistical software package.

Phytochemical analysis of two Hedysarum species

The dynamics of the accumulation of biologically active substances is important for any medicinal plant, since it allows us to estimate the optimal time for harvesting raw materials. During expedition researches samples of above-ground and underground organs of *H. theinum* and *H.neglectum* were taken, after which phytochemical analysis was carried out.

For the phytochemical analysis dry parts of *Hedysarum* (above-ground part and root) were ground as powder in a thresher. Weighed 20 mg of dry powder and prepared an extract for analysis.

Determination of organic compounds in the CO_2 extract.

Sample preparation and analysis methods

4 extracts were taken and analysed by gas chromatography with mass spectrometric detection (7890A/5975C).

Analysis conditions

Sample volume 1.0 µl, sample entry temperature 250°C, no flow splitting. Separation was carried out using a DB-35MS chromatographic capillary column with a length of 30 m, an inner diameter of 0.25 mm and a film thickness of 0.25 µm at a constant carrier gas speed (helium) of 1 ml/min. The chromatography temperature was programmed from 40°C (0 min exposure time) at a heating rate of 5°C/min to 150°C (3 min exposure time) followed by a heating rate of 5°C/min to 280°C (1 min exposure time). Detection was performed in SCAN mode m/z 34–850. Agilent MSD ChemStation software (version 1701EA) was used to control the gas chromatography system, record and process the results and data. Data processing included determination of retention times, peak areas as well as processing of the

spectral information obtained with a mass spectrometric detector. Wiley 7th edition and NIST'02 libraries were used to decipher the obtained mass spectra (total number of spectra in the libraries is more than 550 thousand).

For molecular genetic analysis the following activities were carried out: DNA isolation, determination and editing of nucleotide sequences of ITS markers, phylogenetic analysis using statistical software.

The material for the study was fresh leaves in the flowering phase of H. theinum and H. neglectum collected in Kazakhstan Altai. Fresh leaves of plants growing in a distance of 5–10 m from each other were collected from 60 specimens, 2020–2021. The plant material was preserved in silica gel for further DNA isolation.

DNA isolation and purification: DNA was isolated and purified from *H.theinum* and *H. neglectum* populations DNA was extracted from dried leaf material of the above mentioned *Hedysarum* species. Dellaporta (Dellaporta et al. 1983) methodology was used for DNA isolation.

Determination of DNA quality and quantity:

DNA quality and concentration were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and electrophoresis in 1% agarose gel. For further analysis the DNA concentration was normalised to 20 ng/µl.

Statistical analysis. The ITS nucleotide sequences of two *Hedysarum* species collected in Kazakhstan Altai were aligned with 31 sequences of other species from the NCBI database. MEGA 7 software was used (Kumar et al. 2016). The phylogenetic tree was reconstructed using Neighbor-Joining (NJ) method program (Saitou and Nei 1987) with 1000 bootstrap replications. Haplotype diversity (Hd) and nucleotide diversity (S) were evaluated using the DnaSP6 program (Rozas et al. 2017). Haplotype analysis was calculated using the Neighbor-Joining algorithm in SplitsTree4 (Huson and Bryant 2006). A phylogenetic tree was constructed using PAST version 3.26 (Hammer et al. 2001).

Research Results

The study of morphological parameters of *H. theinum* and *H. neglectum* species allowed us to reveal their adaptation to growing conditions, there are some morphological, physiological and biochemical features, even for closely related species of the genus *Hedysarum* growing in certain soil and climatic conditions.

Characteristic structure of *H. theinum*: greenish stems, plant height averages 60–70 cm, the species is similar to *H. neglectum* in colouring of flowers, but differs in height, *H. theinum* is 30–40 cm higher, also in structure and size of leaves, when cut the root of *H. theinum* is brown-red, with a pleasant smell, while *H. neglectum* is white, but odourless.

Life forms in the community are represented by shrubs, bushes, semi-shrubs and herbaceous forms. The distribution of plants vertically occurs in 2 tiers, Figs. 2–3.

Evaluation of anatomical parameters H. theinum **and** H. neglectum

Anatomical research allows a deeper understanding of the plant under the influence of various factors and in the combination of external and internal structure. The significance of studying the morphogenetic patterns of leaf evolution considers that the adaptation of plants to different environmental conditions is often associated with changes in leaf structure occurring at the morphological and anatomical levels. The study of the leaf anatomical structure as the most plastic organ of plants deserves great attention, since the peculiarities of the structure of different leaf tissues allow both tracing conservative structures and revealing adaptive traits that have evolved in plants during adaptation to modern conditions.

Anatomical structure of the leaf of *H.theinum.* The leaf is covered on both sides by the epidermis. The cells of the upper epidermis are cutinized, consisting of densely compressed cells, rounded in shape. Beneath the upper epidermis, barrel-shaped idioblasts and multilobate cells are revealed closer to the lower epidermis. The mesophyll is divided into columnar and spongy. The palisade parenchyma is located below the upper epidermis, the cells of the spongy mesophyll are located above the lower epidermis. Middle vein strongly convex downwards, the main conductive bundle consists of xylem and phloem, the bundle collaterally closed. Numerous inclusion cells with tannins are found in the bundle lining and above the lower epidermis, Fig. 4A, 2-Table.

Anatomical structure of the leaf of *H.neglectum.* Leaf dorsoventrally, palisade parenchyma located beneath upper epidermis, spongy parenchyma located under palisade parenchyma, Fig. 4B, 2-Table. The leaf is covered on both sides with cells of the covering tissue by epidermis, the cells of the upper epidermis differ in their smaller size from those of the lower epidermis, the lower epidermis shows large, colourless cells, presumably motor cells. Epidermal cells on both sides are strongly cutinized, consisting of small, tightly packed, colourless cells. Columnar mesophyll consists of two rows of palisade parenchyma, cells elongated rectangular, tightly adjoining each other. The cells of the spongy mesophyll are more loosely connected, with large intercellular spaces. The main vein contains a large conductive bundle, consisting of a top directed xylem and a down-directed phloem. The bundle lining is clearly visible, the cells having inclusions, in the form of tannins. Most of the tannins accumulated in the lining cells surrounding the conductive bundles. Above the conductive bundle, colourless cells of the parenchyma, which divides the columnar mesophyll and is located below the upper epidermis, are clearly visible. Sclerenchyma is well developed beneath the phloem, which adjoins the lower epidermis.

Table 2
Morphometric data of the leaf blade of the two species *Hedysarum*

Species	Thickness of the upper epidermis, µm	Thickness of the lower epidermis, µm	Thickness of the mesophyll, µm		Length of vascular bundle, µm	
			Columnar	Spongy	Xylem	Phloem
Hedysarum theinum	23.68 ± 4.3	28.59 ± 1.5	144.19 ± 2.3	120.14 ± 9.6	78.85 ± 11.7	51.64 ± 18.9
Hedysarum neglectum	16.48 ± 3.4	18.57 ± 0.1	127.40 ± 6.5	131.20 ± 8.7	62.33 ± 23.3	49.27 ± 15.9

In comparing the leaves of the two species, the changes were reflected in the transition from the typically dorsiventral leaf structure of *H. theinum* to the isolateral-palisade mesophyll structure of *H.neglectum*. In both species, barrel-shaped idioblasts and multilobed cells were found.

Anatomical structure of the stem of *H. theinum.* The stem is covered with epidermal cells. The epidermal cells are strongly cutinized. Parenchyma, chlorenchyma, collenchyma and sclerenchyma cells are found in the primary cortex. Above the central cylinder there are multifarious cells with inclusions exclusively of tannins. In the central cylinder, the conductive bundles are arranged in a circle, bast fibres are well developed above the phloem, and a continuous layer of cambium can be clearly distinguished between the xylem and phloem. The main difference in the stem of *H.theinum* is that there is a continuous sclerenchymal ring, which is underneath the cambium layer, Fig. 5A. The continuous sclerenchyma ring consists of numerous rows of sclerenchyma cells, i.e. it forms a thick ring. In the centre of the stem are the medullary cells, which consist of thin-walled, transparent cells, they are multifaceted and rounded.

Anatomical structure of the stem of *H. neglectum*. A cross-section of the stem is covered with the epidermis, whose cells are strongly cuticular, consisting of small, rounded cells. The central cylinder occupies more space than the primary cortex. The fascicles are circular, collaterally open with the cambium, the cambium being conspicuously wispy only. Above the phloem the bast fibres are well developed. Xylem consists of numerous rows of vessels. Tannins are found mainly in the bundle wrappers and in the phloem. Pith collapsed, stem hollow, Fig. 5B, 3-Table.

Table 3
Morphometric data of the stem of the two species *Hedysarum*

Species	Thickness Stem, µm	Thickness of the cuticle, µm	Thickness of epidermis, µm	Length of vascular bundle, µm		The thickness of the bast fibre, µm
				Xylem	Phloem	
Hedysarum theinum	650.68 ± 14.3	19.59 ± 1.5	27.37 ± 1.1	107.55 ± 12.5	38.85 ± 18.9	48.59 ± 0.94
Hedysarum neglectum	587.48 ± 13.4	17.57 ± 0.17	22.87 ± 3.5	95.33 ± 13.3	29.71 ± 10.7	54.15 ± 0.30

Figure 6 shows the anatomical structure of the root of *H.theinum.* Unlike the annually renewed above-ground organs, the root grows annually and as a result changes its anatomical structure. The root in cross-section is covered by the periderm of the secondary covering tissue, which is formed from the phylogenic formation tissue. The plant is perennial, in this respect, the primary bark is destroyed and the secondary bark is formed. In the cortex zone, the pith or parenchyma rays are well expressed. The central cylinder contains xylem and medullary rays. The cambium is between the xylem and phloem. The sclerenchyma is well developed. There are xylem vessels in the centre.

Figure 6 shows the anatomical structure of the root of *H. neglectum.* The root on the section is covered by the periderm of the secondary covering tissue, which consists of living phellogen cells, phelloderm and dead phelloderm cells. Secondary cortex is expressed beneath the periderm, and there are xylem and medullary rays in the central cylinder. The cambium is between the xylem and phloem. The sclerenchyma is well developed. There are large xylem vessels in the centre. Large 3–5 xylem vessels are concentrated and alternate with smaller xylem vessels. Root morphometric data of two *Hedysarum* species showed that periderm thickness was *H. theinum* 43.54 ± 13.3 μm, *H. neglectum* 51.41 ± 18.4 μm, root diameter in *H. theinum* 680.68 ± 15.3 μm, *H.neglectum* 697.48 ± 21.7 μm. Cambium thickness in *H. theinum* 86.59 ± 10.5, in *H.neglectum* 83.57 ± 9.17.

Results Of Phytochemical Analysis:

Although a systematic study of the chemical components of *Hedysarum* has been carried out to provide taxonomic evidence for the genus and to support the pharmacological uses of several species within the genus, little data is available on the chemical components of *H. theinum* and *H. neglectum.* Therefore, we present the results of a detailed chemical analysis of extracts from the underground and above-ground parts of the two species from the Kazakhstan Altai Mountains. The results can be used for a variety of human and animal health and nutritional applications. The analysis from the root extract of the two species showed that *H. theinum* has 29 compounds out of a total of 40 components. Although only 15 compounds are peculiar to both species. In H. neglectum, the methylpyruvate is six times greater than in H. theinum. Only *H. theinum* detected levoglucosenone which is a highly functionalized chiral compound, levoglucosenone is used as a building block in organic synthesis to produce a wide range of natural and non-natural compounds. The lactone y-oxybutyric acid (GHB) depressant used as a psychoactive agent predominates in H. neglectum, also this plant has almost twice as many disaccharides consisting of two monosaccharides: glucose and fructose than in *H. theinum*. In *H. theinum* the phenol butanone is detected, which has a sweet, berry and floral taste, butanone being undetectable in *H. neglectum*. Also, in *H. theinum* the glycoside ethyl α-d-glucoside is present in a multiplicative amount of 45.23% and a negligible content of only 0.8% is detected in *H. neglectum*. In *H. theinum*, the highest quantity of vitamin E was found in 11.36% and the plant steroid gamma-sitosterol in 9.2%, but these components were absent in *H. neglectum*. Phytol, an acyclic chemical compound whose basis is made up of isoprene residues, is also a part of *H. theinum*, in the other species has not been found. In both species, squalene,

which participates in metabolism and in the synthesis of steroids and cholesterol, belonging to the group of carotenoids, was found. A comparative phytochemical analysis of the root of the studied species is shown in Table 4.

Table 4
Percentage of compounds, % of root of two *Hedysarum* species

№	Compound	*Hedysarum theinum*	*Hedysarum neglectum*
1	Propanoic acid, 2-oxo-, methyl ester	1,01	6,5
2	Formamide, N-methoxy-	-	0,6
3	Levoglucosenone	0,49	-
4	2-Furanmethanol	-	1,0
5	Methane, trimethoxy-	0,19	-
6	2-Propanol, 1,1-dimethoxy-	-	1,3
7	2-Cyclopenten-1-one, 2-hydroxy-	0,26	-
8	1,2-Cyclopentanedione	-	2,1
9	2-Hydroxy-gamma-butyrolactone	0,23	6,0
10	2,5-Dimethyl-4-hydroxy-3(2H)-furanone	0,22	-
11	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	0,57	1,7
12	2-Propanone, 1-(acetyloxy)-	-	0,8
13	Cyclopropyl carbinol	0,24	1,4
14	Benzeneethanol, 4-hydroxy-	0,54	-
15	4-Cyclopentene-1,3-dione	-	1,3
16	Sucrose	5,29	9,1
17	2-Butanone, 4-(4-hydroxyphenyl)-	3,07	-
18	Octadecanoic acid	1,50	-
19	Ethyl α-d-glucopyranoside	45,23	0,8
20	Benzenepropanol, 4-hydroxy-α-methyl-, (R)-	1,95	-
21	3-O-Methyl-d-glucose	2,88	4,5
22	2-Methoxy-4-vinylphenol	-	1,4
23	Hexadecanoic acid, ethyl ester	1,17	4,5
24	Dibutyl phthalate	5,26	9,4
25	1-Hexadecanol	-	6,0
26	Ethyl Oleate	0,20	1,3

№	Compound	*Hedysarum theinum*	*Hedysarum neglectum*
27	2-(3,4-Dimethoxyphenyl)-6-methyl-3,4-chromanediol	-	0,6
28	9,12-Octadecadienoic acid, ethyl ester	1,55	14,2
29	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	1,40	14,4
30	5,9-Dodecadien-2-one, 6,10-dimethyl-, (E,E))-	-	0,3
31	2-Propenoic acid, 3-(3,4,5-trimethoxyphenyl)-, methyl ester	0,43	3,3
32	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	4,50	-
33	S-Indacene-1,7-dione, 2,3,5,6-tetrahydro-3,3,4,5,5,8-hexamethyl-	0,19	1,8
34	2(5H)-Furanone	-	0,7
35	Ethyl tetracosanoate	0,35	1,5
36	Squalene	0,68	0,6
37	Docosanoic acid, ethyl ester	-	1,4
38	Vitamin E	11,36	-
39	γ-Sitosterol	9,20	-
40	Medicarpin	-	1,7

In the above-ground (stem, leaf, fruit) organs of the two *Hedysarum* species studied the total number of components was 74, of which 55 compounds are found in *H. theinum* and 57 in *H.neglectum*. In both species 37 components were found. in *H. neglectum* 11 components have significant contents, in *H. theinum* 9 components contain significant percentages, the highest 22.49% correspond to monoterpenes (9,10-dimethyltricyclo [4. 2.1.1 (2.5)]decane-9,10-diol) and 15.61% in *H. neglectum*. in both the species the highest phytol content 13.96–15.28, Table 5.

Table 5
Percentage of compounds, % of the above-ground organs of the two *Hedysarum* species

№	Compound	*Hedysarum theinum*	*Hedysarum neglectum*
1.	Propanoic acid, 2-oxo-, methyl ester	0,87	0,95
2.	Pyrimidine, 2-methyl-	0,53	-
3.	N-(2-Methylbutylidene)isobutylami	0,66	0,25
4.	9,12-Octadecadienoic acid, ethyl ester	-	2,33
5.	1-Butanamine, 2-methyl-N-(2-methylbutylidene)-	0,48	0,16
6.	1-Propanamine, 2-methyl-N-(2-methylpropylidene)-	0,47	0,39
7.	Ethanol, 2-(9,12-octadecadienyloxy)-, (Z,Z)-	-	0,29
8.	Pyrazine, 2,5-dimethyl-	0,16	0,18
9.	1-Butanamine, 2-methyl-N-(2-methylbutylidene)-	0,31	0,16
10.	Hexanoic acid, 2-tetrahydrofurylmethyl ester	0,45	0,32
11.	Fumaric acid, ethyl 2-methylallyl ester	-	0,29
12.	Methylamine, N-(1-butylpentylidene)-	0,24	-
13.	Butanoic acid, 4-hydroxy-	0,17	0,28
14.	Heptacosane	-	1,19
15.	N-Butyl-tert-butylamine	0,22	-
16.	Butyl 9,12,15-octadecatrienoate	-	0,29
17.	2-Hydroxy-gamma-butyrolactone	0,14	0,21
18.	cis-p-mentha-1(7),8-dien-2-ol	0,33	-
19.	Diisooctyl phthalate	-	0,22
20.	Cyclohexanol, 2,6-dimethyl-	0,17	-
21.	Phytol, acetate	-	0,46
22.	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	0,31	0,66
23.	Oxirane, 2,2-dimethyl-3-(3,7,12,16,20-pentamethyl-3,7,11,15,19-heneicosapentaenyl)-, (all-E)-	-	0,69
24.	1-Butanol, 3-methyl-, acetate	1,68	-
25.	1-Propanone, 1-phenyl-	0,27	0,22
26.	Benzofuran, 2,3-dihydro-	0,30	0,32

№	Compound	*Hedysarum theinum*	*Hedysarum neglectum*
27.	Acetamide, 2-(5-ethyl-4H-1,2,4-triazol-3-ylthio)-N-(5-methyl-3-isoxazolyl)-	-	0,51
28.	1H-Pyrrole-2,5-dione, 3-ethyl-4-methyl-	0,45	0,30
29.	1H-Pyrrole-2,5-dione, 3-ethenyl-4-methyl-	0,16	-
30.	2-Methoxy-4-vinylphenol	0,25	0,36
31.	2-Ethylbutyric acid, eicosyl ester	0,26	-
32.	N-Phenethyl-2-methylbutylidenimine	0,19	-
33.	Benzene, (1-nitroethyl)-	0,46	-
34.	2-Piperidinemethanamine	-	0,17
35.	(Hexahydropyrrolizin-3-ylidene)-acetaldehyde	0,20	0,22
36.	trans-β-Ionone	0,53	0,42
37.	3-Buten-2-one, 4-(2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-1-yl)-	0,42	0,38
38.	Nanofin	-	0,14
39.	1,3-Benzenediol, 5-pentyl-	0,23	0,36
40.	Sucrose	1,85	4,10
41.	2,5-Dimethyl-4-hydroxy-3(2H)-furanone	-	0,29
42.	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)-	1,47	1,40
43.	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-	0,33	-
44.	Pyrazine, methyl-	-	0,28
45.	3',5'-Dimethoxyacetophenone	0,23	0,53
46.	3-Methyl-4-phenyl-1H-pyrrole	0,51	0,54
47.	Benzeneacetaldehyde	-	0,17
48.	4-Hydroxy-β-ionone	0,31	-
49.	2-Hexadecene, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	0,53	4,44
50.	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	6,05	4,09
51.	α-D-Glucopyranoside, methyl	5,81	-
52.	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	2,90	4,09

№	Compound	*Hedysarum theinum*	*Hedysarum neglectum*
53.	1,2-Cyclopentanedione	-	0,24
54.	2-Pentadecanone, 6,10,14-trimethyl-	0,58	0,80
55.	Hexadecanoic acid, ethyl ester	6,61	12,21
56.	1-(1'-pyrrolidinyl)-2-propanone	-	0,26
57.	9,10-Dimethyltricyclo[4.2.1.1(2,5)]decane-9,10-diol	22,49	15,61
58.	4-Cyclopentene-1,3-dione	-	0,14
59.	Dibutyl phthalate	8,75	10,50
60.	Oxazolidine, 2,2-diethyl-3-methyl-	-	0,21
61.	Phytol	15,28	13,96
62.	Ethyl Oleate	0,28	0,44
63.	Octadecanoic acid, ethyl ester	1,52	-
64.	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	2,54	4,20
65.	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	1,46	-
66.	Retinol, acetate	0,39	0,37
67.	4,8,12,16-Tetramethylheptadecan-4-olide	0,22	0,26
68.	Phthalic acid, di(2-propylpentyl) ester	0,30	-
69.	Phytol, acetate	0,33	9,27
70.	Squalene	0,61	1,00
71.	Vitamin E	5,47	0,37
72.	2,2,4-Trimethyl-3-(3,8,12,16-tetramethyl-heptadeca-3,7,11,15-tetraenyl)-cyclohexanol	0,51	-
73.	Tetratetracontane	0,57	-
74.	1-Butanol, 3-methyl-, formate	-	1,80

Detected and identified: a group of phytosteroids, a number of aromatic and heterocyclic compounds and vitamins. For example retinyl acetate is a natural form of vitamin A, which is the acetate ester of retinol. It has potential antitumor and chemopreventive activity and the antioxidant properties of Vitamin E are also due to the ability of the mobile hydroxyl of the chromane core of its molecule to interact directly with free oxygen radicals and free radicals of unsaturated fatty acids. A comparison of the phytochemical composition of the two studied species reveals significant differences in quantity and quality. The data

obtained can be used for the species identification of *H.theinum* and *H.neglectim*, which has so far been difficult due to the lack of clear systematic morphological characters.

Results of molecular genetic analysis

Sequencing reactions for two plant species of each species were performed using BigDye Terminator v.3.1Cycle Sequencing Kit (Applied Biosystems, USA) with the same primers and separate forward and reverse reactions. Nucleotide sequences were analysed on an ABI 3130 DNA sequencer (Applied Biosystems, USA). For sequencing samples using an ITS (internal transcribed spacer) marker, the isolated DNA was diluted to a working concentration, 100 ng/µl. For sequencing, 20 samples per population were selected, samples 1–3, 1–5, 3 – 1, 3–5 for *H.theinum* and samples 1–3 and 1–4 for *H.neglectum* (Fig. 20). PCR amplification was performed in a thermocycler (Veriti, Thermo Fisher Scientific, USA) for 35 cycles consisting of denaturation at 94°C for 30 s, annealing for 45 s and elongation at 72°C for 1 min 30 s. PCR products were excised from the gel and purified, PCR amplification with forward and reverse primer was performed using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) and according to the manufacturer's temperature schedule. ITS nucleotide sequence determination was performed using a Genetic Analyser 3130 (Applied Biosystems, USA). DNA quality and quantity results from 3 population of *Hedysarum* was given in the Table 6.

Table 6
DNA quality and quantity results from 3 population of *Hedysarum*

№	Sample	Nucleic Acid	260/280	260/230
1	*Hedysarum theinum* 1–1	643,5	2	1,61
2	*Hedysarum theinum* 1–2	788,6	1,99	1,66
3	*Hedysarum theinum* 1–3	962,2	1,95	1,62
4	*Hedysarum theinum* 1–4	771,2	2,05	1,74
5	*Hedysarum theinum* 1–5	1076,8	2,06	1,83
6	*Hedysarum theinum* 2 – 1	786,7	2,04	1,96
7	*Hedysarum theinum* 2–2	588,8	1,97	1,56
8	*Hedysarum theinum* 2–3	774	2,12	1,96
9	*Hedysarum theinum* 2–4	703,5	2,01	1,86
10	*Hedysarum theinum* 2–5	953,6	2,05	1,92
11	*Hedysarum neglectum* 1–1	426,2	2,01	1,48
12	*Hedysarum neglectum* 1–2	586,9	1,97	1,51
13	*Hedysarum neglectum* 1–3	538,2	2,01	1,71
14	*Hedysarum neglectum* 1–4	547	1,99	1,64
15	*Hedysarum neglectum* 1–5	600,5	1,97	1,54

Also, electrophoresis in 1% agarose gel was performed to check DNA quality. The results of the electrophoresis of the 2 populations of the *H.theinum* and the population of *H.neglectum* are shown in Fig. 7.

Sequencing Samples Using Barcoding Markers

Sequencing of *H. theinum* and *H. neglectum* samples using a barcoding marker was carried out.

The polymerase chain reaction of DNA fragments with ITS primer (Internal transcribed spacer, ITS 1, 5.8 S, ITS 2) was performed in 25 µl reaction medium containing 0.2 mM of each dNTP, 250 µM of each primer, 0.5–1.5 mM $MgCl_2$, 1 units of Taq polymerase, 100 ng of DNA, 1 x Taq buffer. Taq polymerase, 100 ng DNA, and 1 x Taq buffer. Internal transcribed spacer 1 and 2, 5.8 S rRNA were amplified using primers ITS1nF: 5'-AGAAGTCGTAACAAGG TTTTCCTGTAGG-3' and ITS4nR: 5'-TCCTCCGCTTATTGATGC-3', primer annealing temperature 58^0 C (White et al. 1990).

PCR amplification was performed on a Veriti thermoamplifier (Applied Biosystems, USA). PCR amplification products were separated electrophoretically in 2% agarose gel, in Tris-EDTA-borate buffer at pH 8.0 and visualized using ethidium bromide using a Bio-Rad gel-documentation system, Fig. 8.

Two samples per population were selected for sequencing, samples 1–3, 1–5, 3 – 1, 3–5 for *H.theinum* and 1–3 and 1–4 for *H.neglectum*. PCR products were excised from the gel and purified; PCR amplification with forward and reverse primers was performed using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) and according to the manufacturer's temperature schedule. ITS nucleotide sequence determination was performed using a Genetic Analyser 3130 (Applied Biosystems, USA).

Phylogenetic Analysis Using Molecular Genetic Markers

A phylogenetic analysis of *H. theinum* and *H. neglectum* species was carried out using molecular genetic markers (Figs. 9). Phylogeny of *Hedysarum* species based on ITS sequences. Alignment of ITS sequences of each species collected in Kazakhstan Altai revealed no intraspecific polymorphism. Phylogeny of *Hedysarum* species based on ITS sequences. The ITS nucleotide sequences of individual specimens of *H. theinum* and *H. neglectum* were submitted to NCBI GenBank. They were aligned together with the sequences of 31 *Hedysarum* specimens from NCBI, while *Astragalus* L. - *Astragalus nezaketiae, A. viciifolius, A. caricinus, A. spatulatus* were selected as outgroup species. The aligned length of ITS sequences was between 609 and 619 bp; the alignment length was reduced to 598 bp when outgroup species were used. Variability was 18% and 82% similarity among samples collected in Kazakhstan. Between the remaining samples from the base the variability was 36.5% and the similarity was 63.5%. The taxonomy of *H. theinum* and *H.neglectum* has been determined to belong to the section *Hedysarum*, class: Magnoliopsida Brongn., subclass: Magnoliidae Novák ex Takht., superorder: Rosanae Takht., order: Fabales Bromhead., Family: Fabaceae Lindl., genus: *Hedysarum* L., species: *Hedysarum theinum* Krasnob. and *Hedysarum neglectum* Ledeb.

ITS nucleotide sequences were aligned and edited in MEGA 7. A phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987) in MEGA 7 (Kumar et al. 2016).

To clarify the phylogenetic relationships of genus *Hedysarum*, nucleotide sequences of *Hedysarum* collected in Eastern Kazakhstan were compared with nucleotide sequences of 41 samples from NCBI (National Center for Biotechnology Information) database and of them 4 samples from collection of Laboratory of Molecular Genetics, Institute of Plant Biology and Biotechnology (Fig. 10).

NJ analysis based on ITS sequences. NJ analysis was performed by transforming ITS sequences. The relationships between haplotypes are represented as a network diagram or splitting graph (Fig. 21). Neighbor Joining analysis identified one major clade of Hedysareae corresponding to clades in the tree topology reconstructed using NJ method haplotypes were identified for ITS region in *Hedysarum* and

outgroup accessors in the cleavage graph analysis, where diversity was 0.6398 and nucleotide diversity (π) was 0.4974, respectively.

For a detailed analysis of Kazakhstan *Hedysarum* species, a separate tree was constructed based on ITS nucleotide sequences. Species of *H. theinum* and *H. neglectum* from Kazakhstan and all available specimens of these species from NCBI were included in the phylogenetic analysis.

Discussion

In Kazakhstan, the genus *Hedysarum* consists of 38 species, without a description of *H. theinum*, as the species *H. theinum* was described later in 1985 by Krasnoborov, so it is not included in the flora of Kazakhstan published in 1966. Thus, the total number of Kazakhstan species of *Hedysarum* is about 20% of the total genus. *H. theinum* is endemic to the Altai Mountains of Kazakhstan. According to the last classification, the species is a ubiquitous shrinking species, belongs to category – 2 vulnerable, declining ranges at risk of extinction due to human activities, rank narrowly local endemics or sub-endemics of the Altai. The priority of studying, preservation and rational use of plant biodiversity is a current trend in our country. In Kazakhstan, during the years of independence, several strategies related to the problems of ecology and environmental protection have been developed. At the present time there are widely presented researches concerning the complex study of individual species of promising plants. Especially widely represented are directions related to study of phytochemical composition and genetic diversity. The results obtained contribute to the development and improvement of the method of phytochemical analysis of plant raw materials *H. theinum* collected from natural populations of Eastern Kazakhstan. According to the Kotukhov's study the stem height of *H.theinum* reaches up to 140 cm and 0,7 cm thickness with 12–14 knots and large leaves, 5 cm length and up to 1,3 cm width. Western Altai plants are smaller in size: 45–70 cm tall and with smaller leaves: about 2 cm long and 0.6-1.0 cm wide.

H. theinum is similar to H. neglectum, with which it is often mixed, and from which it differs in root structure and chemical composition, short, dense multifarious inflorescences. Also with shorter pedicels, longer bracts, bracteoles reaching the apex of calyx teeth, filiform teeth of larger calyx, larger flowers with a boat-like flower rounded on the lower front margin and fruit with a broad margin. According to A.V. Polozhii (1972), *H.theinum* is a relic of the glacial epoch, isolated from the older boreal-forest *H.alpinum.* The experimental data obtained on the general phytochemical screening of the studied phytopreparations showed that the key type of biologically active substances are condensed tannins. The presence of other phenolic compounds: xanthones, flavonoids was also shown. The phytochemistry of the roots of *H. theinum* and *H. neglectum* have been shown to have only minor concentrations of the xanthone mangiferin. Phytosteroids, derivatives of pyran, pyridazine, morphine, phthalic acid, azulene, porphyrin and its analogs and some other compounds were found in plants of genus *Hedysarum* for the first time. The key group of biologically active substances of *H.theinum* and *H.neglectum* condensed tannins was determined.

The cytogenomic characterization of twenty specimens from eight species was performed to assess the genomic diversity and relationships within the *Hedysarum* section. The chromosome variability based on intra- and interspecific variation carrying 45S and 5S rDNA clusters was observed in the studied specimens and four major karyotype groups were identified: (1) *H. arcticum, H. austrosibiricum, H. flavescens, H. hedysaroides* and *H. theinum* (one 45S rDNA and one 5S rDNA chromosome pair); (2) *H. alpinum* and one *H. hedysaroides* specimen (one 45S rDNA and two 5S rDNA pairs); (3) *H. caucasicum* (one pair of chromosomes with 45S rDNA and one pair of chromosomes with 5S rDNA and 45S rDNA); (4) *H. neglectum* (two pairs with 45S rDNA and one pair with 5S rDNA). The species-specific chromosomal markers found in the karyotypes of *H. alpinum, H. caucasicum* and *H. neglectum* may be useful in taxonomic studies of this section (Zviagina, et al. 2013, Yurkevich, et al. 2021). According to the literature, the phylogenetic difference between the two species *H. theinum* and *H. neglectum* was 59 percent (Nuzhdina, et al. 2018).

The reasons for the rarity of *H. theinum* are related to biotic and anthropogenic factors. Among the biotic factors, the leading one is the ecological factor, while the main anthropogenic factor is the collection of *H. theinum* root by local inhabitants for traditional medicine. Therefore, there are currently recommendations to include the species in the Red Data Book.

Conclusion

The anatomical structure of the stem of *H. theinum* has a thick sclerenchymal ring after the cambium, which is absent in *H.neglectum*. The leaf thickness of *H.neglectum* is greater than that of *H.theinum.* Although the leaf length of *H.theinum* is rather longer than that of *H.neglectum.*

Phytosteroids, a number of aromatic and heterocyclic compounds, vitamins and a number of other compounds have been found in plants of the genus *Hedysarum.* The key group of biologically active substances of *H. theinum* and *H. neglectum* was determined. The most important differences in chemical composition (fatty acids, oxycinnamic acids, flavonoids etc.) which allow species identification of *H. theinum* and *H. neglectum* have been established. Flavonoids are natural colouring agents, food antioxidants, tannins, they have antimicrobial action.

The work revealed that the above-ground organs of *H. theinum* accumulates trace amounts of phytol 15.28%, dimethyltricyclodecanediol − 22.49%, vitamin E -5.47%, compared with H. neglectum shows that the plant is medicinal, has anti-inflammatory, antithrombotic and vasodilating properties. Accordingly, the presence of vitamin E in *H. neglectum* is only 0.37%.

The underground part of *H. theinum*, i.e. the root, contains the highest amount of ethyl α-d glucopyranoside − 45.23%, which in *H. neglectum* is only 0.8%. An identification probability of 90–92% showed in the roots that *H. theinum* contains 11.36% tocopherol, which is not found in the other species. There is also squalene, which inhibits the growth of cancer cells, removes heavy metals, mitigates the side effects of radio-chemotherapy, prevents diabetes, has antioxidant properties, normalizes blood pressure, reduces the risk of heart attacks and strokes, strengthens the immune system. In this study, the

results showed that *H. theinum* is indeed a very valuable medicinal plant that should be cherished and multiplied for the production of dietary supplements and medicines.

DNA was isolated from three populations of the plant genus *Hedysarum* L.: 40 samples of plant material from two populations of *H. theinum* and 20 samples of one population of *H. neglectum.* The application of ITS allowed to separate the analysed two species of *Hedysarum* into one major clade, which correspond to the tribe Hedysareae. The phylogenetic tree generated was in good agreement with existing phylogenetic classifications from the NCBI database. Two local species, *H. neglectum* and *H. theinum*, were assigned to the *Hedysarum* section. The results confirmed the polyphyletic origin of the genus.

Abbreviations

H
Hedysarum
ITS
Internal transcribed spacer
NCBI
National Center for Biotechnology Information
DNA
Deoxyribonucleic acid
NJ
Neighbor-Joining
MEGA
Molecular Evolutionary Genetics Analysis.

Declarations

Authors' contributions

Conceptualization, AK and MK; methodology, MK; software, TK and AS; validation, AS; formal analysis, AK; investigation, MK, TK; resources, AK; data management,IZ; writing—original draft preparation, MK and MH; writing—reviewand editing, MK; visualization, IZ; supervision, MK and MH; project administration,MK. All authors read and approved the final manuscript.

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Availability of data and materials

The data used and analyzed in this study can be provided from the respectiveauthor for scientifc, non-proft purpose.

Ethics approval and consent to participate

Not applicable, the study involves no human participants.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

References

1. Artemov, I. A. (2018). Flora of Belukha nature park (Altai republic). Vestnik Tomskogo Gosudarstvennogo Universiteta, Biologiya, (42), 69-101. doi:10.17223/19988591/42/4
2. Cherepanov S.K (1995). Vascular plants of Russia and neighboring states (within the former USSR). - St. Petersburg, - 990 p.
3. Dellaporta S.L., Wood J., Hicks J.B. (1983). A plant DNA mini preparation: Version II // Plant Molecular Biology Reporter.– Vol.1. – P. 19-21.
4. Dolgova A.A., Ladygina E.Ya (1977). Guide to practical classes in pharmacognosy. - M.: Medicine, - 255 p.
5. Dorogina, O., Karnaukhova, N., Agafonova, M.. (2009). Relationships between the Variability of Electrophoretic Profiles of Seed Polypeptides and Ecological-Geographic Conditions of the Habitats of Populations of *Hedysarum theinum* (Fabaceae). Contemporary Problems of Ecology - Contemp Probl Ecol. 2. 506-509. 10.1134/S1995425509060022.
6. Erst, A. A., Zheleznichenko, T. V., Novikova, T. I., Dorogina, O. V., & Banaev, E. V. (2014). Ecological and geographic variability of *Hedysarum theinum* and features of its propagation in vitro. Contemporary Problems of Ecology, 7(1), 67-71. doi:10.1134/S1995425514010053
7. Fedorova, Y. S., Kulpin, P. V., Kotova, T. V., Denisova, S. V., & Beregovykh, G. V. (2021). Study of the antidepressant properties of some plants. Paper presented at the AIP Conference Proceedings, 2419, doi:10.1063/5.0069670
8. Flora of Kazakhstan. - Alma-Ata, Vol. 1-9. - P.1956-1966.
9. Hammer, O., Harper, D. A. T., Ryan, P. D. (2001). PAST: Paleontological statistics software package for education and data analysis. Palaeontol. Electron, 4 (1), 9.
10. Huson, D. H., Bryant, D. (2006). Application of phylogenetic networks in evolutionary studies. Molec. Biol. Evol., 23 (2), 254–267.

11. Karnaukhova, N. A. (2016). Anatomo-morphological features of the leaves of *Hedysarum theinum* (*Fabaceae*) in western altai. Contemporary Problems of Ecology, 9(3), 349-354. doi:10.1134/S1995425516030057
12. Karnaukhova, N. A., Dorogina, O. V., & Selyutina, I. Y. (2018). The anatomical structure of leaf in species of *Hedysarum* SECT. gamotion basin. in south siberia. [Turczaninowia, 21(4), 150-160. doi:10.14258/turczaninowia.21.4.15
13. Karnaukhova, N. A., Selyutina, I. J., & Syeva, S. Y. (2021). Reproductive biology of *Hedysarum theinum* (Fabaceae). Botanicheskii Zhurnal, 106(6), 556-566. doi:10.31857/S0006813621060065
14. Korchagin A.A. Species (floristic) composition of plant communities and methods of its study (1964). // Field geobotany, M.-L.: Nauka, Vol. 3. - pp. 39-62.
15. Krasnoborov I.M., Azovtsev G.R., Orlov V.P. (1985). A new species of the genus *Hedysarum* (Fabaceae) from Southern Siberia Bot. magazine, 70(7): 968-973.
16. Kumar S., Stecher G., and Tamura K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33:1870-1874.
17. Kuminova A.V.(1960). Vegetation cover of Altai. Novosibirsk : 1-450
18. Kupriyanov A.N., Sheremetov S.A., Baykov K.S. (2003). List of higher plants of the Altai-Sayan ecoregion. Biological diversity of the Altai-Sayan ecoregion. Kemerovo: 30-126
19. Lakin G. F. (1990). Moscow: Nauka publ., - 352 P.
20. Litvinenko Yu.A. (2012). methodological recommendations for laboratory work on the discipline "chemistry of natural biological active substances and phytopreparations". - Almaty, Kazakh University, - 85 P.
21. Muzychkina R. A., Korulkin D. Yu., Abilov Z A.(2011). Technology of production and analysis of phytopreparatov. - Almaty, Kazakh university, - 120 P.
22. Nuzhdina, N. S., Bondar, A. A., & Dorogina, O. V. (2018). New data on taxonomic and geographic distribution of the trnLUAA intron deletion of chloroplast DNA in *Hedysarum* (Fabaceae L.). Russian Journal of Genetics, 54(11), 1282-1292. doi:10.1134/S1022795418110108
23. Polozhiy A.V. 1972. To the knowledge of the history and development of modern flora in Yenisei Siberia. History and development of flora and vegetation of Eurasia. L.:136-144.
24. Poniatovskaya V.M. Accounting for abundance and features of species placement in natural plant communities // Field Geobotany, M.-L.: Nauka, Vol. 3. - 1964. - pp. 209-299.
25. Prozina M.N. Botanical microtechnics. - M.: Higher School, 1960. - 206 p
26. Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J.C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S. E., Sánchez-Gracia, A. (2017). DnaSP 6: DNA sequence polymorphism analysis of large data sets. Mol. Biol. Evol., 34 (12), 3299–3302.
27. Saitou N. and Nei M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

28. Shcherbakov A.V., Mayorov A.V. Field study of flora and herbarization of plants. - M.: Publishing House of Moscow State University, 2006. - 84 p.
29. Sviridova T. P., Zinner N. S., 2011. Prospects for growing *Hedysarum alpinumHedysarum theinum*Krasnob. in the conditions of the Tomsk region // Bulletin of Tomsk State University. Biology, pp. 5-16.
30. White T.J., Bruns T., Lee S., Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand D, Sninsky JJ, White TJ, editors. PCR Protocols: a guide to methods and aplications. San Diego: Academic Press; 1990. pp. 315–322.
31. Yurkevich, O. Y., Samatadze, T. E., Selyutina, I. Y., Romashkina, S. I., Zoshchuk, S. A., Amosova, A. V., & Muravenko, O. V. (2021). Molecular cytogenetics of eurasian species of the genus Hedysarum l. (Fabaceae).*Plants, 10*(1), 1-15. doi:10.3390/plants10010089
32. Zhmud, E. V., Zinner, N. S., & Dorogina, O. V. (2020). Trypsin inhibitor activity in representatives of the genus *Hedysarum* (Fabaceae): The polyvariance of seasonal dynamics in southern siberia. Proceedings on Applied Botany, Genetics and Breeding, 181(3), 25-31. doi:10.30901/2227-8834-2020-3-25-31
33. Zinner, N. S., Nekratova, A. N., Shchukina, A. V., & Kovaleva, A. L. (2021). Biological features of high altitude rare medicinal plant species *Hedysarum theinum*Krasnob. in western siberia cultivation. Acta Biologica Sibirica, 7, 77-85. doi:10.3897/abs.7.e67122
34. Zviagina, N. S., & Dorogina, O. V. (2013). [Genetic differentiation of altai-sayan endemic *Hedysarum theinum* (Fabaceae) evaluated by inter-simple sequence repeat analysis]. Genetika, 49(10), 1183-1189. doi:10.7868/s0016675813100135

Figures

Figure 1

Point of collection of *Hedysarum,*

A- *Hedysarum theinum*, B- *Hedysarum neglectum*

Figure 2

Coenopopulation of H. theinum

Figure 3

Coenopopulation of H. neglectum

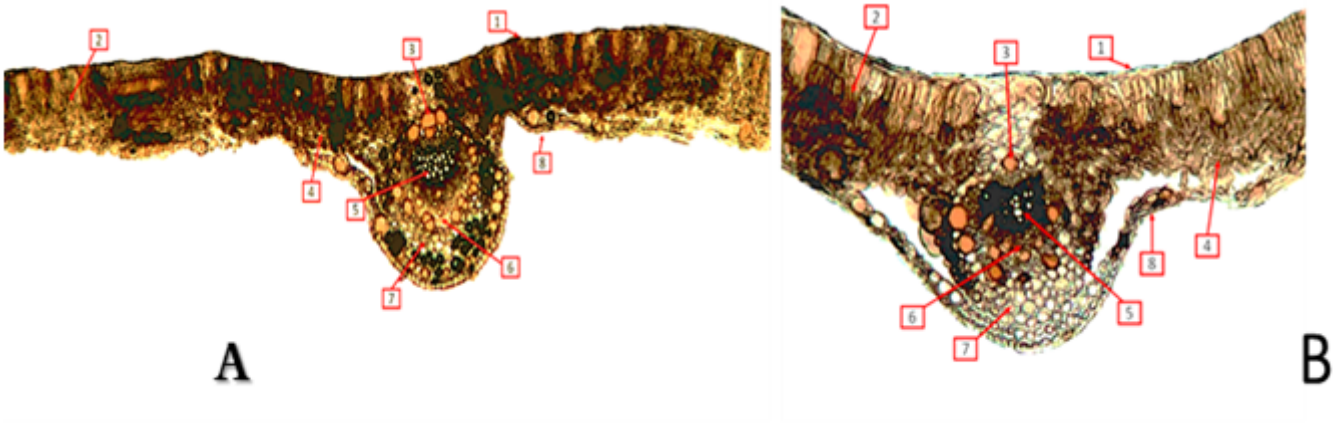

Figure 4

Cross-section of the leaves

A - Anatomical structure of the leaf blade of *Hedysarum theinum.*

1 - Upper epidermis; 2- Columnar mesophyll; 3- Inclusions; 4- Spongy mesophyll; 5- Xylem; 6- Phloem; 7- Parenchyma; 8- Lower epidermis.

B- Anatomical structure of the leaf blade of *Hedysarum neglectum,*

1- Upper epidermis; 2- Columnar mesophyll; 3- Inclusions; 4- Spongy mesophyll; 5- Xylem; 6- Phloem; 7- Parenchyma; 8- Lower epidermis.

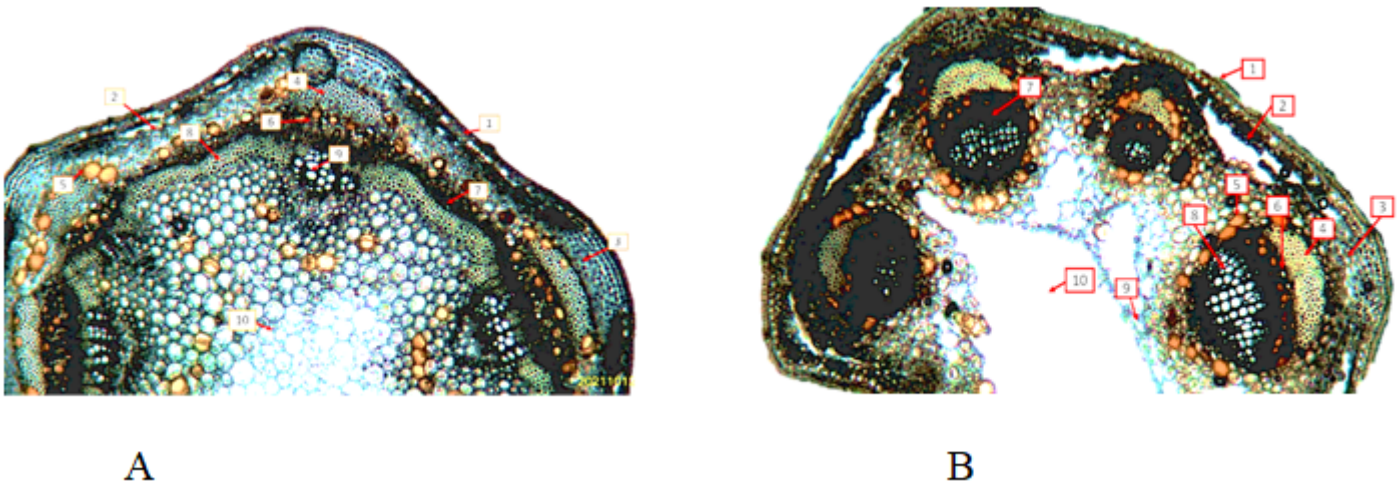

Figure 5

Cross-section of the stem

A-Anatomical structure of the stem of *Hedysarum theinum.*

1 - Epidermis; 2- Primary cortical parenchyma; 3- Collenchyma; 4- Fibers; 5- Inclusions; 6- Phloem; 7- Cambium; 8- Sclerenchyma; 9- Xylem; 10- Pith.

B - Anatomical structure of the stem of *Hedysarum neglectum.*

1- Epidermis; 2- Primary cortex parenchyma; 3- Collenchyma; 4- Bast fibres; 5- Inclusions; 6- Phloem; 7- Bundle cambium; 8- Xylem; 9- Parenchyma; 10- Cavity.

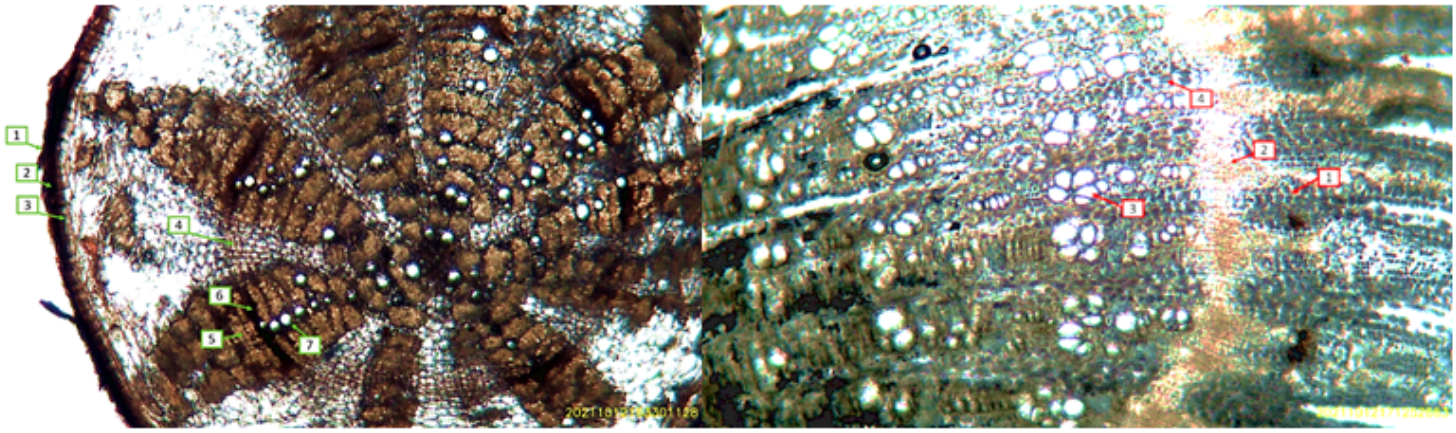

Figure 6

Cross-section of the root

A-Anatomical structure of the root of *Hedysarum theinum.*

1 - Phellema; 2 - Phellogen; 3 - Phelloderm; 4 –Ray; 5 - Phloem; 6 - Cambium;

7 - Xylem.

B - Anatomical structure of the root of *Hedysarum neglectum.*

1- Phloem; 2- Cambium; 3- Xylem. 4- Ray.

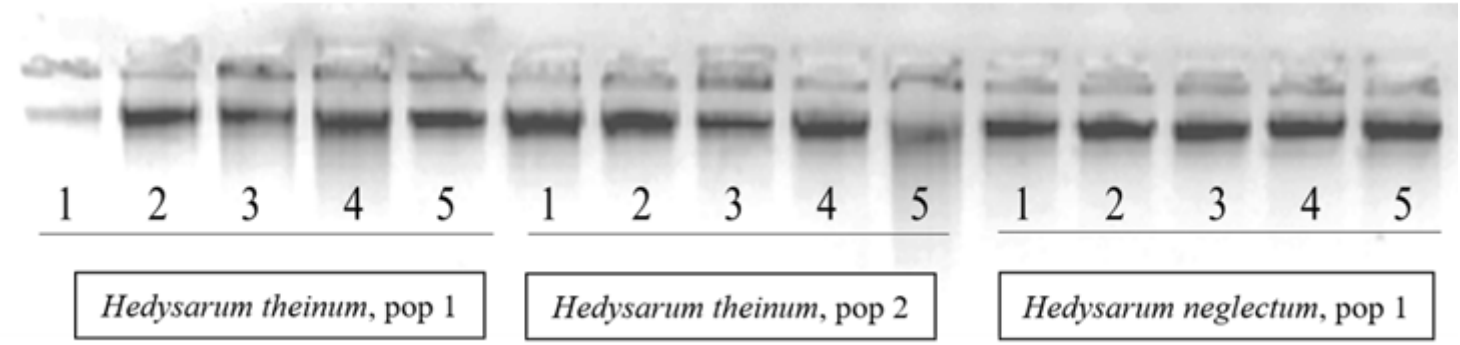

Figure 7

Results of electrophoresis of the *Hedysarum*population

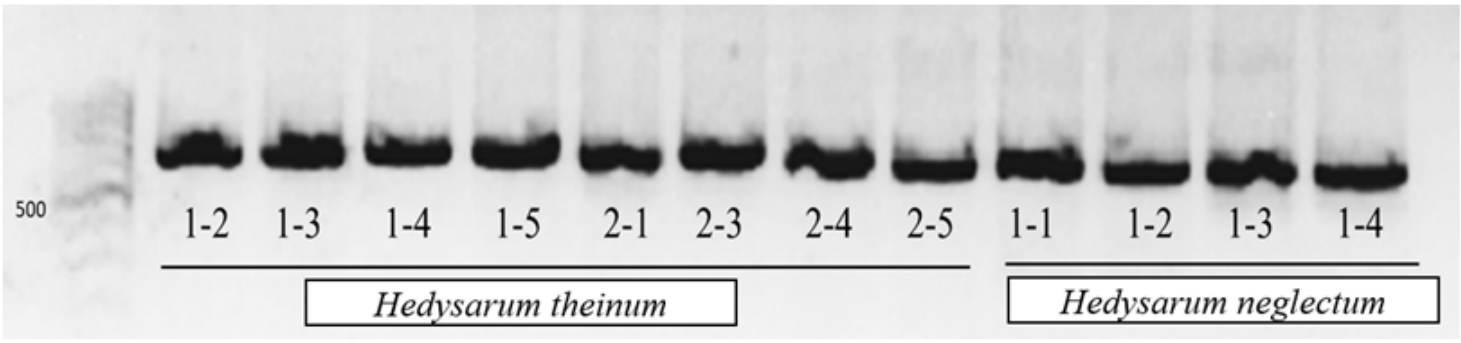

Figure 8

Agarose gel of PCR products from samples of *H.theinum* and *H. neglectum* by ITS marker

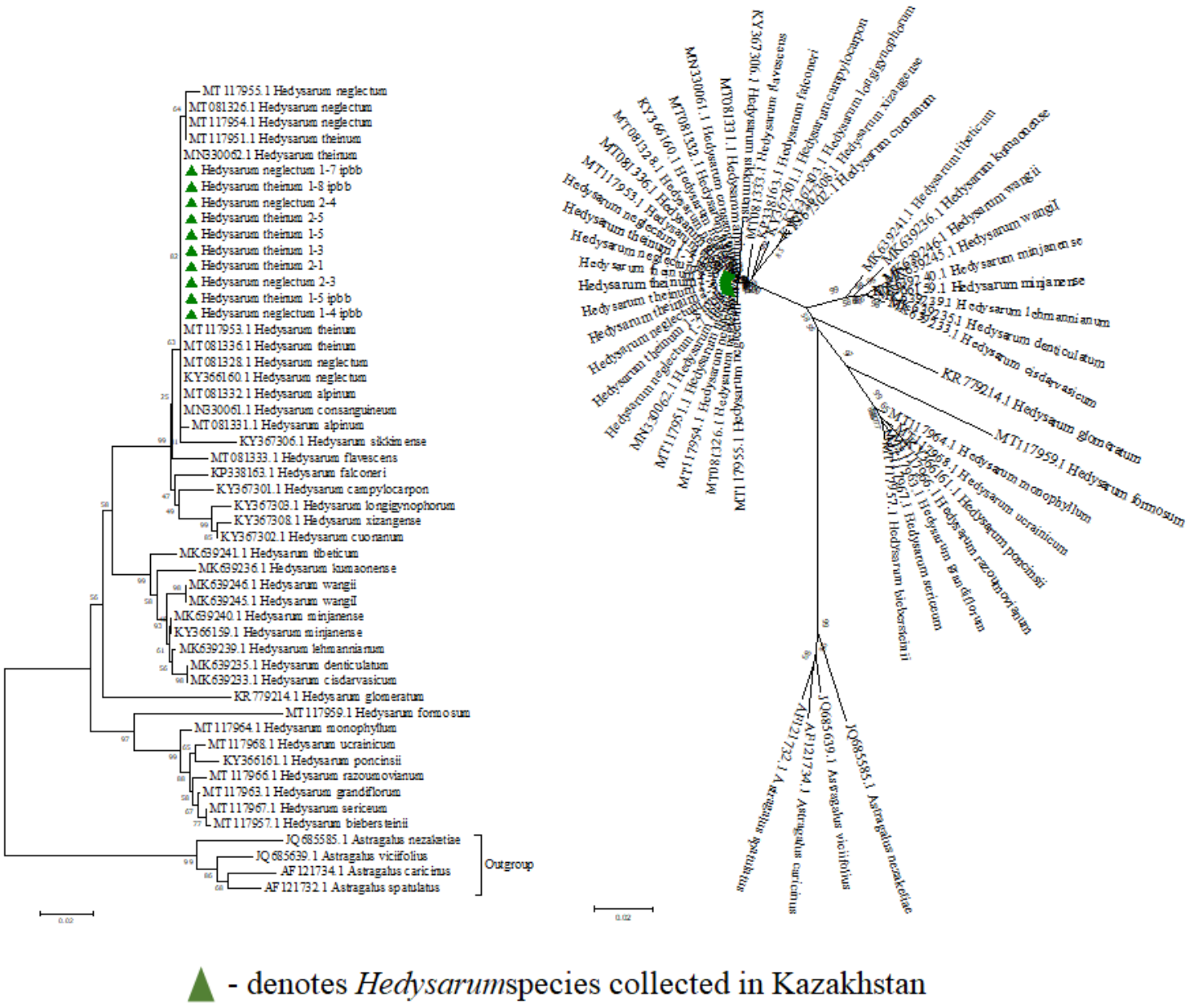

Figure 9

Phylogenetic tree of Neighbor-Joining species of the genus *Hedysarum* based on ITS sequencing

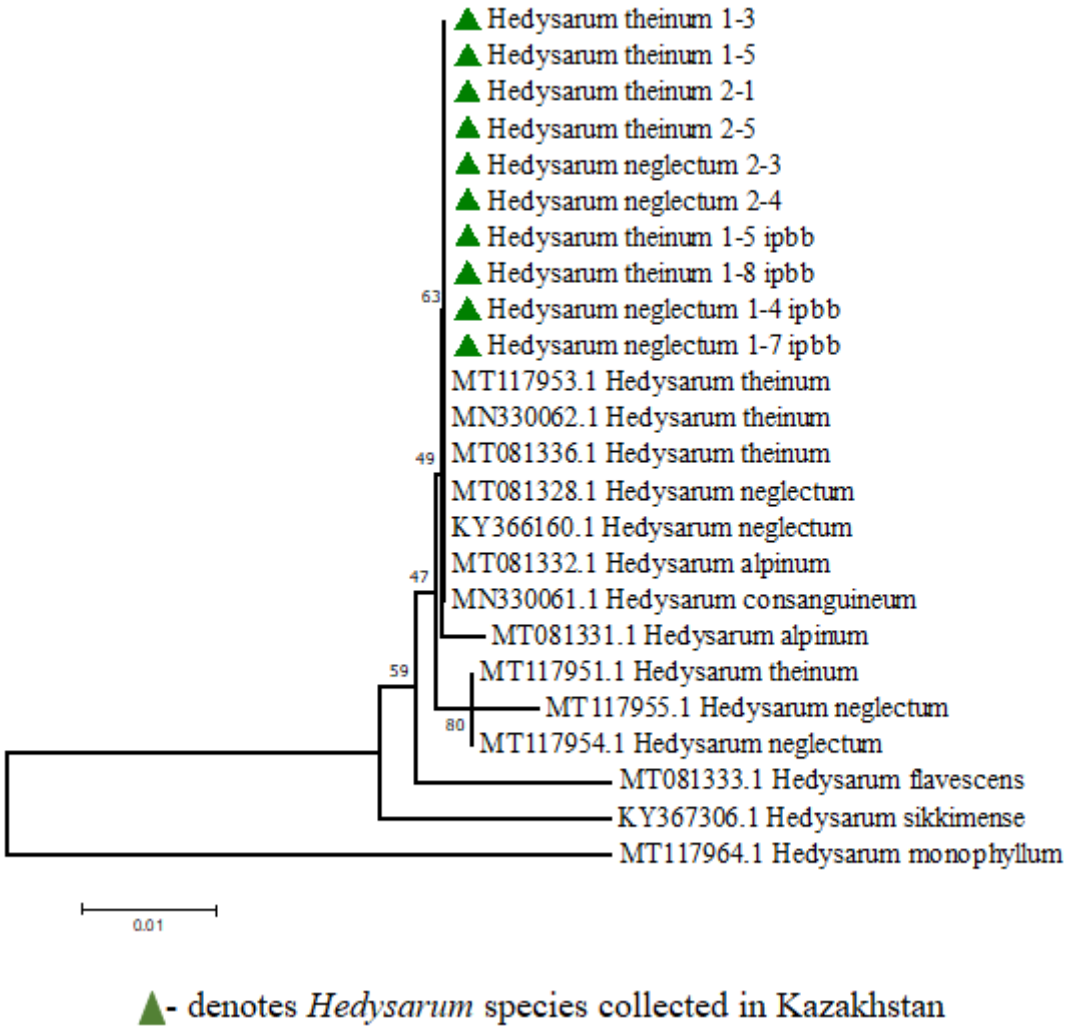

Figure 10

NJ phylogenetic tree of *H. theinum* and *H. neglectum* species based on ITS sequencing

Supplementary Files

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- APPENDIX.docx