



# Genotyping NS and NSK herbaria specimens of *Hedysarum* (Fabaceae) using DNA sequencing

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## ABSTRACT

Over 2000 specimens of the genus *Hedysarum* L., belonging to 48 species and 4 subspecies, were digitised and placed into CSBG SB RAS Digital herbarium whilst making a digital inventory of the herbarium collections of the Central Siberian Botanical Garden (NS, NSK). Totally, 466 nucleotide sequences were determined for 136 voucher specimens using the Sanger sequencing method. DNA sequences were obtained for 25 *Hedysarum* taxa for the first time.

**Keywords:** biodiversity, digital herbarium, GBIF, NS, NSK herbarium collections, taxonomy, vascular plants, ITS, ETS, *trnL-F*, *rpl32-trnL*, *rbcL*, *psbA-trnH*

## РЕЗЮМЕ

Нуждина Н.С., Ковтонюк Н.К. Генотипирование образцов рода *Hedysarum* (Fabaceae) методом секвенирования ДНК в гербариях NS и NSK. Более 2000 гербарных образцов, принадлежащих 48 видам и 4 подвидам рода *Hedysarum*, были оцифрованы и размещены в Цифровом гербарии ЦСБС СО РАН при инвентаризации гербарных коллекций Центрального сибирского ботанического сада (NS, NSK). Для 136 ваучерных образцов были определены 466 последовательностей ДНК методом прямого секвенирования по Сэнгеру. Для 25 таксонов рода *Hedysarum* нуклеотидные последовательности определены впервые.

**Ключевые слова:** биоразнообразие, цифровой гербарий, GBIF, гербарные коллекции NS, NSK, таксономия, сосудистые растения, молекулярные маркеры, ITS, ETS, *trnL-F*, *rpl32-trnL*, *rbcL*, *psbA-trnH*

Preserved specimen collections are the most important source of scientific information about the distribution of species in the past and present, which allows simulation of the dynamics of objects in the future. An herbarium sample can reliably confirm the presence of a plant in a specific place at a certain time (Kovtonyuk et al. 2020). Herbarium collections and the data they hold are valuable, not only for the traditional studies of taxonomy and systematics, but also for ecology, bioengineering, conservation, food security and the human social and cultural elements of scientific collection (James et al. 2018). Free and open access to biodiversity data is an essential for informed decision-making to achieve conservation of biodiversity and sustainable development (Penev et al. 2017). Digital inventories of herbarium collections and the publication of up-to-date datasets and catalogues through the Global Biodiversity Information Facility (GBIF 2022) allows researchers worldwide to view and work remotely with collections and biodiversity data (Gatilova et al. 2021).

The *Hedysarum* L. is one of the largest genera of the tribe *Hedysareae* in Fabaceae (Duan et al. 2015). There are ca. 200 species of perennial herbaceous species of *Hedysarum* distributed worldwide, mainly in Eurasia, North Africa and North America (Yakovlev et al. 1996, Liu et al. 2017a,b).

A few *Hedysarum* species occur in Northern Asia: 22 taxa are known for Siberia (Pjak & Ebel 2000, Nikiforova 2005, Kurbatskij 2006), and 13 for Russian Far East (Pavlova

1989). Five species and one subspecies are known to be common: *H. alpinum* L., *H. branthii* Trautv. & C.A. Mey., *H. dasyarpum* Turcz., *H. gmelinii* subsp. *setigerum* (Turcz. ex Fisch. & C.A. Mey.) Kurbatski, *H. inundatum* Turcz., and *H. vicioides* Turcz. Due to ecological adaptation to different environmental conditions, some species of *Hedysarum* form a component of variable types of vegetation: *H. gmelinii* Ledeb., *H. alpinum*, and *H. hedysaroides* ssp. *arcticum* (B. Fedtsch.) P.W. Ball. Several *Hedysarum* species are endemic: *H. chajrakanicum* Kurbatski, *H. latibracteatum* N.S. Pavlova, *H. sachalinense* B. Fedtsch., *H. zundukii* Peschkova, *H. turczaninovi* Peschkova and *H. tschuense* Pjak & A.L. Ebel. Geographically, areas of rare *Hedysarum* species are highly restricted and their local populations are often under the pressure of negative environmental or anthropogenic factors like overgrazing, mining of limestone, unlicensed collection of medicinal herbs, etc. (Kurbatskij 2006).

With the progress in genotyping and DNA barcoding of plants in the last two decades, nucleotide sequences retrieved from nuclear or chloroplast nucleic acids have become an important source of information contributing to classification and to reconstruction of the phylogenetic relationships between plant species (Shaw et al. 2005, Shneyer 2009, Dong et al. 2012, Shekhovtsov et al. 2019). DNA analysis gives researchers the opportunity to analyze the intra- and inter-population genetic variability, improving our strategy for the conservation of rare and endangered plant

species with application to *in vitro* microclonal propagation technologies (Nuzhdina & Dorogina 2012, Muraseva et al. 2017). Molecular studies also provide valuable data to assess the level of differentiation between morphologically similar congeners and to estimate the possibility of hybridization processes between them (Suprun & Schanzer 2012, Nuzhdina et al. 2020). Sequence data are often used for inferring the phylogeography of the taxa and the direction of migration processes (Mikhaylova et al. 2010), and to conduct the analysis of haplotypes in order to study the consequences of ice age on relict species distribution and refugium locations (Shen et al. 2005).

Lots of new *Hedysarum* species were described from the European part of Russia, Iran and China (Pjak & Ebel 2000, Sa 2007, Ranjbar et al. 2008, Knyasev 2011, Dehshiri & Goodarzi 2016). In addition to eco-morphological features and geographic area, some authors provide molecular data supporting the status of newly described species (Liu et al. 2017a, 2019, Nafisi et al. 2019, Juramurodov et al. 2021).

The purpose of the current study is to conduct the genotyping of *Hedysarum* herbarium collections at the Central Siberian Botanical Garden (Novosibirsk, Russia). In this context, we want to achieve the following objectives: (1) to extract genomic DNA in *Hedysarum* specimens belonging to herbarium collections NS and NSK (herbarium codes hereafter follow Thiers 2022); (2) to determine the nucleotide sequences for two nuclear ribosomal (ETS and ITS) and four chloroplast markers (*trnL-F*, *matK*, *rbcL*, and *psbA-trnH*); (3) to deposit the molecular data in the GenBank (National Center for Biotechnology Information, NCBI); (4) to determine the efficiency of nuclear (ETS, ITS) and chloroplast (*trnL-F*, *rp32-trnL*, *rbcL*, *trnH-psbA*) molecular data in genotyping the herbarium specimens of *Hedysarum* taxa.

## MATERIAL AND METHODS

In the NS and NSK herbarium collections (USU 440537) are 2005 *Hedysarum* specimens, all of which were digitised according to international standards at 600 dpi using special scanners ObjectScan 1600 (Microtek, Taiwan) and provided with individual barcodes containing herbarium acronyms and serial numbers. Every image is provided with a colour scale and a scale bar. Digital copies of all scanned *Hedysarum* specimens are available at CSBG SB RAS Digital Herbarium (<http://herb.csbg.nsc.ru:8081>) and published as a dataset (Kovtonyuk et al. 2022) on the GBIF portal. Names of taxa conform to the World Checklist of Vascular Plants (WCVP 2022). Sampling was designed to represent the taxonomic diversity in the genus *Hedysarum* and included 136 voucher specimens belonging to 48 species and 4 subspecies. Total genomic DNAs were extracted using either CTAB method (Doyle & Doyle 1987) or the NucleoSpin Plant II DNA extraction kit (Macherey & Nagel, Germany). The DNA concentration and purity were determined spectrophotometrically using a BioSpectrometer kinetic and  $\mu$ Cuvette G1.0 (Eppendorf, Germany). Primers used for amplification and sequencing were: ETS<sub>Hedy</sub> and 18S-IGS (Liu et al. 2017b) for ETS, ITS4 and ITS5 for ITS (White et al. 1990), “c” and “f” for *trnL*-intron and *trnL-trnF* intergenic spacer (Taberlet et al. 1991),

*rp32-F* and *trnL* (UAG) (Shaw et al. 2007) for *rp32-trnL* none-coding region, *trnHGUG* and *psbA* (Shaw et al. 2005) for *trnH-psbA* intergenic spacer, and *rbcL* “F” and *rbcL* “R” (Kress & Erickson 2007) for subunit “a” of *rbcL* gene.

All polymerase chain reactions (PCR) were done in a Thermal Cycler T1000 (Bio-Rad, USA). PCR was performed in a 40  $\mu$ L volume with the following components: 1x reaction buffer, 90 nM MgCl<sub>2</sub>, 11 nM of each primer, 8 nM of each dNTP, 1.5 units of Taq-polymerase. The amplification conditions were 3 min at 94°C, followed by 35–40 cycles of 30 sec – 1 min at 94°C, 30 sec – 1 min at 55–58°C, 45 sec – 2 min at 72°C, and a final extension at 72°C for 7 min. After DNA amplification 4  $\mu$ L of each PCR product was stained with SYBR-Green (ThermoFischer Scientific, United States) and visualized using the gel-documentation system Gel-Doc XR+ equipped with the ImageLab Software Imaging System (Bio-Rad). The remaining volume of PCR products was purified from the PCR components by sorption on AMPureXP magnetic particles (Agencourt, United States) and sequenced directly using the BigDye Ready Reaction DNA Sequencing Kit v.3.1 (Thermo Fischer Scientific). The Sanger reaction products were purified from unincorporated fluorescent dyes by centrifugation (900 g, 2 min) through a column of 750  $\mu$ L of Sephadex G-50 Fine suspension (GE Healthcare, United States), the sequencing products were run on an 3130XL automatic gene analyzers (Applied Biosystems, United States) at the Genomics Shared Use Center (Novosibirsk).

## RESULTS AND DISCUSSION

All digitised specimens of the genus *Hedysarum* stored in NS and NSK herbarium collections were deposited in CSBG SB RAS Digital herbarium (<http://herb.csbg.nsc.ru:8081>). Scientific name, locality, collection date, names of collectors, ecology and revision label were recorded for each specimen. Every voucher specimen was marked by label.

The dataset consists of 2005 specimens of *Hedysarum* collected between 1879 and 2021. The registration of *Hedysarum* specimens in NS and NSK herbaria per year are shown in Figure 1 with the maximum gatherings while preparing on the “Flora of USSR”, published between 1934 and 1964 and the “Flora of Siberia” in 1987–2003.

Most of the *Hedysarum* specimens were collected in Siberia and the Russian Far East and the rest in Eastern Europe, the Caucasus, Central Asia and North America or were transferred from the herbarium collections of Europe as duplicates (Fig. 2).

Herbarium specimens originating from 17 countries, Russia (1816 specimens), U.S.A. (45), Mongolia (40), Kazakhstan (35), Tajikistan (17), Canada (15), Kyrgyzstan (10), Azerbaijan (7), Georgia (5), Turkey (5), Japan (3), China (2), Ukraine (2), Italy (1), Slovakia (1), Turkmenistan (1), and Uzbekistan (1), were sampled.

The earliest herbarium collection examined in the study was 143 years old; it was the specimen of *H. alpinum* var. *americanum* Pursh, collector Ezra Brainerd, 10.07.1879, USA, Western Vermont, Lamoille County, Smuggler’s Notch (NS0042576). The oldest herbarium from which DNA was isolated, amplified and then sequenced successfully was

120 years: *H. cephalotes* Franch. subsp. *pamiricum* B. Fedtsch. (synonym of *H. minjanense* Rech. f.), collected by Fedchenko O.A. and Fedtchenko B.A., 18.07.1901, Tadzhikistan (NS0041722), and *H. alpinum*, collected by Litvinov D.I., 18.08.1902, China (NS0008014).

In the study, genomic DNA was extracted for 136 voucher specimens of *Hedysarum*. PCR products were obtained for all DNA templates except two. In the cases of *H. alpinum* (NS0041572), collected in 1907, and *H. hedysaroides* (L.) Schinz & Thell. (NS0041560), collected in 1933, we could not amplify and sequence due to low concentrations (2.7 ng/μl and 12 ng/μl, respectively) and purities (A260/A280 = 1.01 and 1.42) of genomic DNAs. It also seems to be a degraded DNA since we did not obtain a PCR product for any of tested marker loci (*trnL-F*, *rbcL*, *psbA-trnH* and ITS) at any amount of DNA templates.

Totally 466 nucleotide sequences for 52 *Hedysarum* taxa were determined in the study (see the Table 1). Among them, 25 taxa were sequenced for the first time.

The length of nucleotide sequences determined for *Hedysarum* herbarium collection varied from 255 (*trnH-psbA*) to 693 bp (ITS). The *trnL-F* sequences were of maximum variability. For instance, the circumboreal species, *H. alpinum*, has *trnL-F* sequences with lengths from 323 to 677 bp. As shown, this locus is highly informative in *Hedysarum*, demonstrating the significant genetic polymorphism at inter- and intra-species level and could be treated as evidence of non-monophyletic origin of the genus (Liu et al. 2017b).

The lowest informativeness was shown by the *rbcL* gene – region of chloroplast DNA coded the large subunit of ribulose biphosphate carboxylase. Using this marker locus, we obtained PCR product only from half of all DNA samples (52 %), which was the most non-efficient result in the present study.

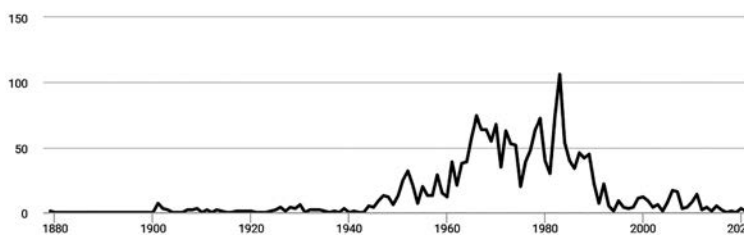
## CONCLUSIONS

The results of analyses based on sequencing of multiple DNA regions clearly show the significance of genotyping of herbarium collection, including old specimens. It was revealed that nuclear ribosomal external transcribed spacers (ETS) and the chloroplast non-coding regions, *trnL-F* and *trnH-psbA*, are the most informative and promising in DNA barcoding of *Hedysarum* species. Based on these markers, nucleotide sequences were determined for most *Hedysarum* taxa (91–98 %), including specimens up to 120 years old. Currently 466 nuclear and chloroplast DNA sequences were obtained in the study.

Among them, 82 nucleotide sequences for *trnL-F* (43), *trnH-psbA* (13), *rpl32-trnL* (13), and ITS (13) marker loci were deposited in the National Center for Biotechnology Information, NCBI. In our study 25 *Hedysarum* taxa were sequenced for the first time. The analyses of different DNA barcodes among and within *Hedysarum* species confirms its high variation and therefore can serve as a useful tool to evaluate divergence in a cryptic species or in phylogenetic reconstruction of the genus in Northern Asia. Indeed, the work is on progress by us.

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**Figure 1.** The registration of *Hedysarum* specimens at NS and NSK herbaria per year between 1879 and 2021



**Figure 2.** Distribution map of *Hedysarum* specimens deposited at NS and NSK herbaria

**Table 1.** The main characteristics of nucleotide sequences determined for *Hedysarum* specimens at NS, NSK herbaria for six marker loci.

| Locus                    | ETS        | ITS       | <i>trnL-F</i> | <i>rpl32-trnL</i> | <i>rbcL</i> * | <i>trnH-psbA</i> |
|--------------------------|------------|-----------|---------------|-------------------|---------------|------------------|
| Number of sequences**    | 125 (97 %) | 18 (90 %) | 125 (91 %)    | 34 (72 %)         | 50 (52 %)     | 114 (98 %)       |
| Length of sequence, b.p. | 349–357    | 689–693   | 320–758       | 565–639           | ≈ 600–700     | 255–415          |

\* PCR products were obtained but not sequenced yet.

\*\* Numbers in brackets are the percentage of sequences obtained to the total amount of matrices tested for this locus.

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