DNA specificity in two Hedysarum sections, *Hedysarum* and *Multicaulia* (Siberia, Russia) inferred from ETS sequence data

Natalia Nuzhdina[1,*], *Abdollah Ghorttapeh* Hassanzadeh[2] and *Olga* Dorogina[1]

[1]Central Siberian Botanical Garden of the Siberian Brunch of the Russian Academy of Sciences, Novosibirsk, Russia
[2] Horticulture Crop Science Research Department, West Azerbaijan Agricultural and Natural Resources Research and Education Center (AREEO), Urmia, Iran

Abstract. Based on sequence data of the nuclear ETS markers of 20 species, the relationships of sections *Hedysarum* and *Multicaulia* were assessed. Phylogenetic analysis was performed on the complete sequences using the Neighbor-Joining methods. The nuclear marker ETS is investigated for the first time in the most species excluding *H. hedysaroides*, *H. inundatum*, *H. ferganense*, and *H. neglectum*. The length of sequences varies from 349 to 357 nucleotides. It is noteworthy that ETS data significantly discriminate *H. ferganense* from both sections, *Hedysarum* and *Multicaulia.* Moreover, our results did not support the subsectional division of *Multicaulia* section. Therefore, the ETS sequence data obtained in our study for 20 taxa of *Hedysarum* evidence the non-monophyly of section *Multicaulia*.

1 Introduction

The genus *Hedysarum* L. (sweet vetch) is the largest of the tribe *Hedysareae* DC., the legume family (Fabaceae L.) and includes up to 200 species of perennial herbs [1, 2]. *Hedysarum* species have been successfully used due to their crop and fertilization importance and as ornamental, melliferous, and medical plants [3].

The monophyletic origin of the genus *Hedysarum* and tribe *Hedysareae* has been discussed [1, 4]. Last decades, nuclear and plastid DNA markers provided evidences concerned with molecular divergence and phylogenetic relationship in plants. The *Hedysarum* genus were re-defined using both nuclear and chloroplast DNA sequences [1]. The latest infrageneric system was performed based on 58 species *Hedysareae* using five nuclear and five chloroplast markers [1]. It was demonstrated by Mironov [5] that pericarp anatomy has a section-specific properties for *Hedysarum* (previously - *Obscura*, or *Gamotion*) and *Multicaulia* sections. Choi and Ohashi [4] believed that sweet vetch species belonging to these sections are characterized by a different chromosome number, x = 7 and x = 8, correspondingly.

* Corresponding author: nsnuzhdina@gmail.com

It was demonstrated that the nuclear DNA marker, external transcribed spacer (ETS) region of the 18S rRNA gene is a useful tool for studying the phylogeny of closely related species. Up to now, previous ETS molecular dating efforts used limited *Hedysarum* taxa sampling. Thus, we want to achieve the following objectives: (1) to access a molecular variability in *Hedysarum* species by sequencing ETS region of a nuclear DNA; (2) to resolve the relationships among the species of two sections of a sweet vetch, *Hedysarum* and *Multicaulia*.

2 Materials and methods

The present study was based on leaf material collected by the first author in 2010 and 2011, and the materials removed from herbarium specimens preserved in NSK herbarium (Novosibirsk, Russia). The samples analyzed in this study were collected from Siberia, Russia excluding *H. hedysaroides* (Italy), *H. ferganense* (Tajikistan) and *H. truncatum* (Kamchatka, Russian Far East).

The analysis included a total of 21 samples representing two sections of *Hedysarum*. DNA was purified from 40 mg of foliar issue using the NucleoSpin Plant II Kit (Macherey-Nagel, USA) according to the manufacturer's protocol. The DNA concentration and purity was determined using a BioSpectrometer kinetic and μCuvette G 1.0 (Eppendorf, Germany). *Corethrodendron fruticosum* (= *H. fruticosum*) was used as outgroup.

Genomic DNA was amplified for ETS, using ETS-Hedy (F) and 18S-IGS (R) primers and the protocol (Liu *et al.* 2017). All PCR reactions were done in a Thermal Cycler T1000 (Bio-Rad, USA). Amplified DNA fragments were stained with SYBR-Green (ThermoFischer Scientific, USA) and visualized in 1.5 % agarose gel using Gel-Doc XR+ documentation system equipped with the ImageLab Software (Bio-Rad, USA).

PCR fragments obtained were then purified by sorption on AMPureXP magnetic particles (Agencourt, USA) and sequenced directly using the BigDye Ready Reaction DNA Sequencing Kit v. 3.1 (Thermo Fischer Scientific, USA). The Sanger reaction products were purified from unincorporated fluorescent dyes by centrifugation (900 *g*, 2 min) through a column packed with 750 μL of the Sephadex G-50 Fine suspension (GE Healthcare, USA) and analyzed on 3130XL automatic genetic analyzer (Applied Biosystems, USA) in the SB RAS Genomics Core Facility (ICBFM, Novosibirsk).

The nucleotide sequences were aligned by Clustal-W algorithm and the Neighbor-Joining trees were computed using the program MEGA 10.0.5. Robustness of the brunches was calculated using 1000 bootstrap replications (BS).

3 Results and discussion

Based on the sequence data of ETS the genetic relationship between two sections of a sweet vetch, *Hedysarum* and *Multicaulia* were assessed (Fig. 1). The length of sequences varied from 349 (H. *consanguineum*) to 357 nucleotides (*H. dahuricum*, *H. dasycarpum*, *H. ferganense*). The molecular data of nuclear ETS marker used were polymorphic and provided substitutions to delimit the most species.

The nuclear ETS marker was investigated for the first time in the most taxa excluding *H. hedysaroides*, *H. inundatum*, *H. ferganense*, and *H. neglectum*. There were no indels among several species from *Hedysarum* section, H. *consanguineum*, *H. arcticum*, and *H. inundatum*, and between *H. alpinum* - *H. vicioides*.

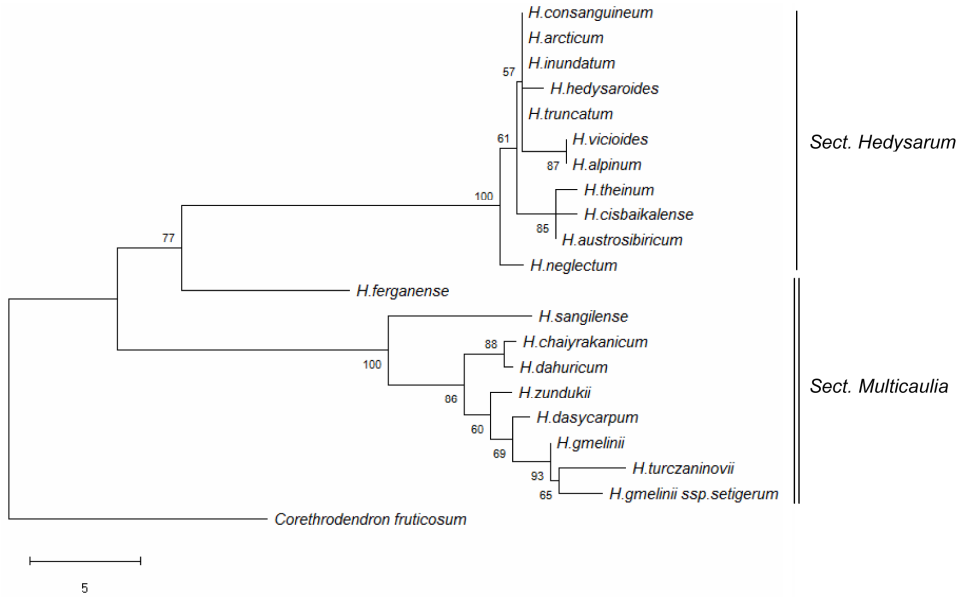

Fig. 1. Phylogenetic relationships among *Hedysarum* species based on Neighbor-Joining method using ETS sequence data. Bootstrap percentages are shown next to the branches.

The nuclear ETS marker allowed us to clarify relationships between two sections, *Hedysarum* and *Multicaulia* with 20 sampled accessions. Results of DNA sequencing presented herein demonstrated that the relationship registered between two sections using ETS sequences was consistent with the *trn*L-F chloroplast data [6].

According to the Neighbour-Joining tree based on ETS sequencing, *Hedysarum* species belonging to different sections, *Hedysarum* and *Multicaulia*, were grouped separately (100 BS). The exception was *H. ferganense* which had an intermediate position between the section; the bootstrap support was of 77%. The separate clade with the single species, mountain legume *H. ferganense*, usual in Altai and Middle Asia, is placed closer to *Hedysarum* section clade rather than *Multicaulia* which the species belongs to.

Among *Multicaulia* section, there was a group of closely related species: two endemics, *H. turczaninovii* and *H. gmelinii* subsp. *setigerum*, and its widespread congener *H. gmelinii*. Hence, the molecular DNA similarity between *H. gmelinii* and its stemless subspecies evidenced from *trn*L-F sequence data previously [6] has been improved. Another two local endemics, *H. chaiyrakanicum* and *H. dahuricum* possessed a high molecular similarity according their ETS nucleotide sequences (88 BS). Both species possess non-typical pale yellow or pinkish flowers and are distributed among steppe vegetation strictly, on stony slopes and rocks.

Our results revealed incongruence between ETS-tree location and subsectional affinities among *Multicaulia* section taxa. Species belonging to *Subacaulia* and *Multicaulia* subsections [4] were dispersed chaotically which has evidenced the unnatural subdivision of *Multicaulia* section, whereas the sequences of *trn*L-F locus of cpDNA analyzed in our previous study [6] have discovered the heterogeneity (biphyly) of section *Hedysarum*. Taking into consideration the intermediate position of *H. ferganense* at ETS phylogenetic tree, we can assume therefore that our data supports the hypothesis of non-monophyletic origin of *Hedysarum* proposed by Liu et al. (2017).

This work was supported by the budgetary project of the CSBG “Assessment of morphogenetic potential of Northern Asian plant populations using experimental methods” (no. AAAA-A17-117012610051-5).

References

1. P.-L. Liu, J. Wen, L. Duan, E. Arslan, K. Ertugrul, Z.-Y. Chang, PlosOne **12**(1) (2017)
2. G. P. Yakovlev, A. K. Sytin, Y. R. Roskov, Legumes of Northern Eurasia: a check-list (Royal Botanical Gardens, Kew, 1996)
3. M. Yu. Vdovichenko, I. N. Kuzovkina, Kh. Petts, B. Shnaider, Fiziol. Rast. **54**(4) (2007).
4. B.-H. Choi, H. Ohashi, Taxon, **52** (2003)
5. Ye. M. Mironov, Bull. Mosc. Soc. Natur. Biol. Ser. **105**(1) (2009)
6. N. S. Nuzhdina, A. A. Bondar, O. V. Dorogina, Rus. J. Genet. **54**(11) (2018)