Genetic relatedness and taxonomy in closely related species of *Hedysarum* (Fabaceae)

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Abstract

A multidisciplinary study, engaging morphological, carpological and molecular data, has been performed to investigate the genetic relatedness and taxonomic boundaries of the close species *Hedysarum gmelinii*, *H. setigerum* and *H. chaiyrakanicum* (Fabaceae) with overlapped distribution areas in southern Siberia. The diagnostic features of these legume species are analyzed and discussed, including their macro- and micromorphological characteristics, seed coat ornamentation and inter-simple sequence repeat (ISSR) profiles. The morphometric features, pod and seed microsculpture traits of *H. chaiyrakanicum* and the ISSR patterns of the three species have been determined for the first time. Sprout, leaf, calyx, corolla, and stem rachis measurements, leaflet indumentum type and ISSR patterns significantly discriminate *H. chaiyrakanicum* from the other two species, whereas plant height, lengths of stem and leaf, and length and width of leaflet show opposite ranges of variation for *H. gmelinii* and *H. setigerum* though none of them is reliable in species identification. Ornamentation of seed coat and ISSR patterns does not differ significantly in the species. Therefore, our study supports the separate taxonomic treatment of *H. chaiyrakanicum* and the subordination of the cryptic species *H. setigerum* within *H. gmelinii*.

Keywords: *Hedysarum gmelinii* complex; Genetic relationships; ISSR markers; Morphology; Seed coat pattern; Molecular taxonomy

1 Introduction

Siberia is the largest part of Northern Eurasia covering a total area of approximately 10 million km^2 between the Ural Mountains, Kazakhstan, China, Mongolia and the Russian Far East (Yakovlev et al., 1996; Malyschev, 2000). The considerable richness of Siberian flora, including over 4500 vascular plant species (Malyschev et al., 2005), is thought to be a result of both the palaeogeological history of plant lineages and the influence of the Pleistocene glaciations. Subsequent postglacial colonization resulted in the recent adaptations of extant species to current ecosystems like mountain ranges, steppes, plains (Revushkin, 1987; Malyschev, 2000). According to Takhtadjan (1986), Holarctic Siberia includes the Circum-Boreal, Irano-Turanian and a part of the Western Asian floristic regions.

Legumes play a key role in cereal- and legume-based systems of semi-arid regions of Siberia and are commonly used to improve the soil organic matter as well as a valuable natural forage crop due to their excellent nitrogen fixation activity and drought resistance (Fedtschenko, 1972; Yakovlev et al., 1996). Despite their large spatial coverage and ecological and economic relevance, few molecular taxonomic studies have been conducted on Siberian legumes.

The genus *Hedysarum* L., represented by 160–200 species of perennial/annual herbs, semishrubs or small shrubs (Yakovlev et al., 1996; Amirahmadi et al., 2014), is the largest genus of the tribe Hedysareae (Fabaceae). In addition to their crop and fertilization importance, *Hedysarum* species have been successfully used as melliferous and ornamental plants in landscape architecture (Fedtschenko, 1972; Yakovlev et al., 1996; Xu and Choi, 2010). The valuable medicinal properties of some *Hedysarum* roots (Krasnoborov et al., 1985) fostered biotechnological analyses of microclonal propagation and *in vitro* plant cell culturing (Vdovitchenko et al., 2007; Erst et al., 2015).

Among approximately twenty *Hedysarum* species distributed in Siberia (Kurbatskij, 2006), *H. gmelinii* Ledeb., assigned to sect. *Multicaulia* Boiss., is the most widespread taxon often used as a green fodder and pasture crop (Fedtschenko, 1972). Its geographic range covers south-eastern Europe, northern and central Asia and China, where it occurs in diverse habitats varying in light intensity, water balance, soil humidity and salinity. Individuals of *H. gmelinii* show a great phenotypic variation, from almost dwarf to long-stemmed plants, with different leaflet numbers, hairiness patterns, and corolla colors (white, pink, violet). Different ploidy levels within a ploidy series of x = 7 and x = 8 chromosome base numbers are known for *H. gmelinii* (e. g., 2n = 14, 16, 28, 32, 48, 56; Cherkasova, 2009; Kurbatskij and Malakhova, 2003; Kurbatskij, 2006; Yan et al., 1989). The varying morphological and karyological characteristics led to a highly complex taxonomic identification of cryptic species or intraspecific taxa within the *H. gmelinii* group.

Since the description of *H. gmelinii* by von Ledebour (1812), several short-stemmed or rosette-type species have been established (Fedtschenko, 1972; Kurbatskij, 1990; Ranjbar et al., 2008; Sa, 2007; Dehshiri, 2013). One of them, *H. setigerum* Turcz., resembles *H. gmelinii* in its pheno-morphological traits, geographic distribution, and karyotype variability (2n = 14, 28, 32, 48; Kurbatskij and Malakhova, 2003; Kurbatskij, 2006), although initially it was classified into a different section, *Subacaulia* Boiss. (Fedtschenko, 1972). Both species are adapted to similar steppe, river banks and rocky habitats in southern Siberia. Different taxonomic revisions have treated *H. setigerum* either as an independent species (Fedtschenko, 1972; Yakovlev et al., 1996) or as a subspecies of *H. gmelinii* (Fedtschenko, 1902; Kurbatskij, 2006), or as its variety (Sa et al., 2010). The third species, strict endemic *H. chaiyrakanicum* Kurbatskij (1990) known only from two localities from Central Tuva, southern Siberia, is phenotypically closely related to *H. setigerum* and could be classified in the same section, *Subacaulia*. *H. chaiyrakanicum* differs from *H. setigerum* in its lighter pink flowers, fewer leaflets and fewer pod (lomentum) segments. Surprisingly, such a strict endemic is characterized by two chromosome numbers, 2n = 14 and 16 (Zvyagina et al., 2016), measured in individuals from a single population (Khairakan Mountain).

Previous taxonomic studies conducted on these Siberian *Hedysarum* taxa using morphological, seed coat sculpturing, and seed anatomical traits (Mironov, 2000; Sa et al., 2010) demonstrated that phenotypic characteristics were varying greatly among the species and that the separation of the taxa from sections *Multicaulia* and *Subacaulia* was unclear. Choi and Ohashi (2003) further subsumed *Subacaulia* as a subsection of sect. *Multicaulia* based on the similar type of morphological (habit, flowers, seeds, pollen) and anatomical characteristics.

Despite several molecular studies of *Hedysarum* aiming to characterize the species and to establish their genetic affinities and phylogenetic relationships, most of these works investigated the Mediterranean and the Middle East *Hedysarum* taxa. Analyses of restriction patterns of the chloroplast and mitochondrial DNAs (Baatout et al., 1985) and RFLPs (restricted fragment length polymorphisms) of nuclear ribosomal DNAs (Trifi-Farah and Marrakchi, 2001) were conducted for *H. carnosum, H. spinosissimum, H. coronarium, H. pallidum* and *H. flexuosum*. ISSR (inter-simple sequence repeat) markers were applied to investigate *H. coronarium* genetic polymorphism (Marghali et al., 2012) and to access the molecular similarity among *Hedysarum* and *Sulla* species (Chennaoui-Kourda et al., 2007). Several phylogenetic studies of *Hedysarum* and *Sulla* taxa and the tribe Hedysareae were based on internal transcribed spacer (ITS) of nuclear DNA (Ahangarian et al., 2007; Chennaoui et al., 2007), or based on plastid *trn*L-*trn*F (Amirahmadi et al., 2010) or *rbc*L sequences (Zitouna et al., 2014), or used multilocus sequencing analysis of ITS, *trn*L-*trn*F and *mat*K regions (Amirahmadi et al., 2014). However, no molecular studies have been performed yet for the *H. gmelinii* complex.

Joint analyses of both morphological and molecular data could be a suitable approach to identify and separate closely related species that might have experienced complex evolutionary processes such as reticulation, introgression or lineage sorting (Duminil and Di Michelle, 2009). A compilation of studies conducted for *Festuca eskia* complex (Torrecilla et al., 2013) and monogeneric Azollaceae family (Pereira et al., 2011) have shown the concerted pattern of morphological and molecular variation used to discriminate the species. The same method was used to evaluate an infra-specific variability level in endangered and endemic species *Ulmus lamellosa* (Liu et al., 2016). The use of different phenotypic and molecular traits has proved to be a valid system for germplasm selection of commercial varieties and agro-ecotypes of the valuable fodder legume *H. coronarium* (Flores et al., 1997) and for assessment of the relatedness between its wild accessions (Ruisi et al., 2011).

The main objective of our study was to conduct a comprehensive analysis of morphological, carpological, and molecular traits in the three close species of the *H. gmelinii* group (*H. gmelinii*, *H. setigerum* and *H. chaiyrakanicum*) aiming to evaluate the genetic relatedness among the species and to outline their taxonomic delimitation.

2 Materials and methods

2.1 Plant material

For analysis 109 accessions of *H. gmelinii*, *H. setigerum* and *H. chaiyrakanicum* were collected from 30 natural populations located in the following regions of southern Siberia, Russia: Tuva, Khakasia, Krasnoyarsk and Irkutsk (Fig. 1, Table 1). Morphological analysis of 21 traits was performed on 30 individuals (14 populations), 20 (2) and 30 (14) of *H. gmelinii*, *H. chaiyrakanicum,* and *H. setigerum,* respectively (Table 1), including five paratype specimens of *H. chaiyrakanicum* (accession nos. 58–62). Analysis of lomentum morphology, seed size, and seed surface sculpture was done for 20 samples of each species. ISSR analysis was conducted for 15 individuals of each species (Table 1). Individual samples of the close congeners *Hedysarum theinum* (sect. *Obscura*) and *H. fruticosum* (sect. *Fruticosa*) and of the less related *Astragalus bifidus* (Fabaceae) were also analyzed and used as outgroups in the molecular study.

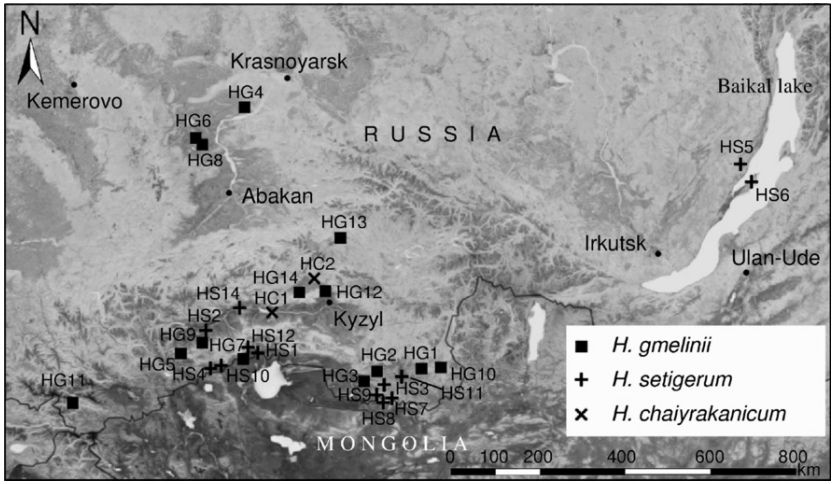

Fig. 1 Geographical distribution of the studied taxa of the *Hedysarum gmelinii* complex in southern Siberia. Symbols map the sampled localities of the studied populations of each taxon (see Table 1).

alt-text: Fig. 1

Table 1 Origins of specimens examined in the phenotypic and molecular study of *H. gmelinii* group species (southern Siberia, Russia). Ind. No., individual number.

alt-text: Table 1

Population code	Location, collectors' names, year of collection	Ind. No.
H. gmelinii		
HG1	SW Tuva, Tchirgalandy 50°28′N 97°E; Sobolevskaya K.A., Sergeeva A. 1946	1–2
HG2	S Tuva, Naryn; Krasnoborov I.M., Kosinetz L. 1972	3–5
HG3	S Tuva, Naryn; Krasnoborov I.M., Kosinetz L. 1972	6–8
HG4	Krasnoyarsk region, Primorsk; Pavlova G., Kilyn F. 1973	9
HG5	C Tuva, Khendelen; Koroleva A., Gontcharova E. 1976	10
HG6	N Khakasia, Shira lake; Zvyagina N., Artemov I. 2011	11–15
HG7	C Tuva, Sap pass 50°44′N 91°50′E; Zvyagina N., Artemov I. 2011	16–23
HG8	N Khakasia, Tzelinny 54°34.402′N 89°54.183′E; Zvyagina N., Artemov I. 2011	24–31
HG9	W Tuva, Tsagan-Shibety range 50°46.742′N 90°11.703′E; Artemov I. 2007	32–33
HG10	C Tuva, Tere-Khol lake 50°36′N 97°25′E; Artemov I. 2007	34–35
HG11	S Tuva, Katunsky range 49°42′N 87°12′E; Artemov I., Kosterin O. 1988	36–37
HG12	C Tuva, the mouth of the Ujuk river 52°06′N 94°15′E; Schaulo D., Krasnikov A. 1987	38
HG13	C Tuva, Ujuksky range, Germanovka; Lomonosova M., Kosinetz L. 1971	39
HG14	C Tuva, Kurtushibinsky range, Khut; Schaulo D., Nalpina T. 1980	40
H. chaiyrakanicum		

HC1	C Tuva, Khairakan Mountain, N slope; Timokhina S., Paschenko S. 1974	41–44
	C Tuva, Khairakan Mountain, 51°30′N, 92°40′E. Krasnoborov I.M. 2007	45
	C Tuva, Khairakan Mountain 51°30′N, 92°55′E. Schaulo D. 1989	46
	C Tuva, Khairakan Mountain, 51°34.474′N, 93°03.784′E Zvyagina N., Artemov I. 2011	48–57
	C Tuva, Khairakan Mountain; Timokhina S., Egorova G. 1974	58–62
	C Tuva, Khairakan Mountain, SE slope; Selyutina I.Yu. 2006	63–64
HC2	C Tuva, Semiozersk 52°15′N, 93°47′E Schaulo D., Schaulo I. 1998	47
H. setigerum		
HS1	SE Tuva, Sagly; Korotkova E., Danilov M. 1977	65–67
HS2	C Tuva, Shekpeer; Koroleva A., Gontcharova E. 1976	68–70
HS3	SE Tuva, 50°08′N 95°55′E; Selyutina I.Yu. 2006	71–75
HS4	SE Tuva, Mugur-Aksu; Selyutina I.Yu. 2006	76–78
HS5	Irkutsk region, Baikal Lake; Karnaukhova N.A. 2005	79–80
HS6	Irkutsk region, Olkhon island; Karnaukhova N.A. 2005	81–83
HS7	S Tuva, Sengilen range 49°59.845′N 96°22.382′E; Artemov I. 2007	84–86
HS8	S Tuva, Sengilen range 49°59.744′N 96°22.567′E; Artemov I. 2007	87
HS9	S Tuva, Sengilen range 50°28.595′N 96°00.627′E; Artemov I. 2007	88–91
HS10	C Tuva, Chandagaity; Ershova E., Yakovleva T. 1978	92–102
HS11	W Tuva, Yttyg-Khaya Mountain; Pshennikova L., Makerova G. 1976	103
HS12	S Tuva, Sagly 50°30′N 91°15′E; Sobolevskaya K.A. 1946	104–107
HS13	Tuva, Kurbatskij V. 1946	108
HS14	C Tuva, the mouth of the Solbelder river; Krasnoborov I., Sakovitch M. 1973	109

2.2 Morphological analysis

Major quantitative and qualitative morphological traits considered to be diagnostic for the studied species according to Fedtschenko (1972) and Kurbatskij (1990) were selected. A total of 21 vegetative and reproductive traits were examined in the individuals of *H. gmelinii*, *H. setigerum,* and *H. chaiyrakanicum*. Sixteen quantitative macromorphological features were studied: length of the most developed axis, LA (cm); length of the most developed stem, LS (cm); length of leaf, LL (cm); number of leaflet's pairs, NL; length of leaflet, LLT (mm); width of leaflet, WLT (mm); length of the most developed sprout, LSP (cm); length of the most developed inflorescence, LI (cm); length of calyx tube, LCT (mm); length of calyx lobes, LCL (mm); length of corolla standard, LSD (cm); length of corolla keel, LK (cm); length of corolla wings, LW (cm); ratio between calyx tube and calyx lobe lengths (T/L); ratio between keel and standard lengths (K/S); and ratio between wings and keel lengths (W/K).

Micromorphological measurements of leaflet trichome length, TL (μm) and trichome density, TD (per mm^2) were taken on both the adaxial (ad) and abaxial (ab) surfaces. Analyses of leaflet hairiness were done using SEM (Hitachi T-1000). Before scanning, specimens were softened by keeping them in Petri dishes containing moistened cotton pellet.

Rachises of last year's leaves and sprouts (R) were for the first time regarded as a taxonomically valuable trait and recorded as multistate (3-states) discrete characteristics: 0 (absence), 1 (few), 2 (abundant).

2.3 Carpological analysis

The lomentum traits were examined using a light microscope Carl Zeiss Stereo Discovery V12 equipped with AxioCam MRc-5 and AxioVision 4.8 software. Lomentum traits, seed size, and seed coat ultrasculpture features were studied under SEM (Hitachi T-1000). The specimens were thawed for 24–72 h by placing them in a chlorophorm/ethanol (1:1) solution before scanning. Mature lomenta and seeds were photographed at several magnifications, length and width of lomentum segments (SGL, SGW) and seeds (SL, SW) were measured and seed coat ornamentation was inspected.

2.4 DNA extraction and ISSR assay

Total genomic DNA was extracted from a set of silica gel dried samples using Nucleospin Plant II kit (Macherey and Nagel, Germany). DNA concentration was quantified spectrophotometrically at 260 nm with the BioPhotometer (Eppendorf, Germany) and its qualities were estimated from the A_{260}/A_{280} ratio.

DNA amplification was achieved in 25 µl of final volume reaction mixture consisting of 2.7 mM $MgCl_2$, 1.25 mM primer, 0.4 mM dNTPs, 1 × PCR buffer, 1.5 units of Taq DNA polymerase (Medigen, Russia), 50 ng of DNA template, and sterile water. The thermocycler program was set to 2 min at 94 °C, 35 cycles of 0.25 min at 94 °C, 0.45 min annealing and 1.30 min extension at 72 °C, and a final 7 min extension at 72 °C. PCRs were carried out with C 1000 Thermal Cycler (BioRad Laboratories, USA). All amplifications were performed 2–5 times in order to recognize the repeatable bands. The amplification products were resolved electrophoretically on 1.5% agarose gel in 1x TBE buffer. The resultant bands were stained with 0.1% SYBR-Green added to the reaction volumes before the electrophoresis. The amplified fragments were visualized and registered using Gel-Doc XR+ documentation system with Image Lab Software (Bio-Rad Laboratories, USA).

2.5 Statistical and genetic analyses

Basic statistics of mean, standard deviation, range of variation, coefficient of variation and p-value (one-way ANOVA test with pairwise comparisons and alpha level of 0.05) were calculated for the morphometric data using PAST software (Hammer et al., 2001). Total deviation was measured as a sum of deviations.

The amplified ISSR fragments were treated as dominant markers and scored as present (1) or absent (0) and converted into binary matrices. Matrices were scored using PAST (Hammer et al., 2001). ~~Nei (1972) genetic distances~~ Genetic distances of Nei (1972) between species pairs were estimated with TFPGA 1.3 (Miller, 1997). The genetic diversities of the species were calculated using Shannon's information index, $H = - \Sigma p_i \log_2 p_i$, where p_i is the frequency of band i when present. Informativeness of bands (Ib) was calculated with the algorithm $Ib = 1 - (2 \times |0.5 - p|)$, where p is the proportion of all accessions containing the band (Prevost and Wilkinson, 1999). The resolving power (Rp) of each primer was determined according to the formula: $Rp = \Sigma Ib$.

Independent multivariate principal component analysis (PCA) and neighbor-joining clustering analyses were performed using PAST. As both PCA and NJ analyses of genetic, morphometric, carpological, and combined input data sets gave congruent clusters, we present the results of PCA conducted for separated and joined data sets, and a NJ dendrogram based on ISSR data as the most informative one.

Genetic ISSR structuring within the individuals of the *H. gmelinii* data set was assayed using AMOVA and Structure 2.3.4 (Pritchard et al., 2000; Earl and von Holdt, 2012). Standard AMOVA and hierarchical AMOVAs for two (*H. gmelinii* + *H. setigerum* vs *H. chaiyrakanicum*) and three (*H. gmelinii* vs *H. chaiyrakanicum* vs *H. setigerum*) taxonomic groups were conducted using Arlequin ver 3.5 (Excoffier et al., 2005). We tested the structure of the data for K = 1–17 groups with Markov chain Monte Carlo simulations of 100.000 iterations after a burn-in of 500.000 generations. Selection of the best grouping was based on the ΔK approach (Evanno et al., 2005) conducted with the on-line application Structure Harvester.

3 Results

3.1 Morphological trait variability

Statistically significant differences ($p < 0.05$) were obtained for most of the studied morphological features in the three studied species (see Supplementary data, Table S1 and Fig. S1). Only the length of inflorescence (LI) and the ratios between calyx tube and lobes (T/L) and between corolla keel and standard (K/S) did not differ significantly (Fig. S2).

The average mean value of stem length (LS) in *H. gmelinii* was statistically significant and maximum among the species analyzed (6.8 ± 6.4 cm), which confirms its potential classification within sect. *Multicaulia*, and, conversely, the classification of the short-stemmed *H. setigerum* and *H. chaiyrakanicum* within sect. *Subacaulia*. However, the variability of stem length in *H. gmelinii* was extremely high (CV = 93.9) and overlapped with those of the other studied taxa in some individuals (e.g., individuals 36 and 37, from the same population (HG3) had stems of 0.5 and 7 cm long, respectively).

The length of the leaves and length of the most developed axis markedly separated *H. setigerum* (LL = 7.7 ± 3.2 cm; LA = 14.1 ± 4.1 cm) from the long-leaved and tall-stem plants of *H. gmelinii* (LL = 11 ± 3.7 cm;

LA = 20.3 ± 7.6 cm) and *H. chaiyrakanicum* (LL = 9.7 ± 2.8 cm; LA = 20.6 ± 6.0 cm). The leaflet size (length, LLT, and width, WLT) was significantly different between *H. gmelinii* (mean 12 × 4.7 mm) and *H. setigerum* (mean 9 × 3.9 mm).

In this study we provide the first data on morphology of *H. chaiyrakanicum.* The endemic *H. chaiyrakanicum* can be characterized by having the fewest number of the leaflets (NL = 3.6 ± 0.7), the larger number of rachises (R = 1.8 ± 0.6), the highest sprout length (LSP = 18 ± 5 cm) and the smallest calyx (LCT = 2.1 ± 0.2 mm; LCL = 4.6 ± 0.9 mm) and corolla lobes (LSD = 1.1 ± 0.1 cm; LK = 1.1 ± 0.8 cm; LW = 0.8 ± 0.1 cm).

Our analyses revealed several diagnostically important morphological vegetative and reproductive characteristics which exhibited species-specific differences in *H. chaiyrakanicum* and allowed it to be separated from *H. gmelinii* and *H. setigerum.* They are the lower number of leaflets (NL), longer sprout length (LS), dense covering of rachises (R), the smallest lengths of petals (LSD, LK, LW) and of calyx tube (LCT) and W/K correlation, and indumentum density of adaxial leaf surface (TDad). Also, the length of calyx lobes, density (TDab) and length of the leaflet trichomes (TLab and TLad) significantly discriminate *H. chaiyrakanicum* from *H. setigerum* (Table S1,Fig. S1).

By contrast, *H. setigerum* and *H. gmelinii* only differed statistically based on the lengths of axis (LA) and leaf (LL) and width (WLT) and length (LLT) of leaflets. Although the length of stem (LS) varied significantly between the two species ($p < 0.05$), the trait could not be used as field identification characteristic with regard to the high coefficient of variation (93.9 in *H. gmelinii* and 71.5 in *H. setigerum*). Moreover, in *H. gmelinii* and *H. setigerum,* all reproductive characteristics varied similarly (Table S1) and could not be treated as a sufficient data source to discriminate the species.

The trichome length and trichome distribution patterns in the taxa of the *H. gmelinii* group are summarized in Table S1. Scanning electron microscopy shows that there is a common trichome type in all examined species. The three species show trichomes with non-glandular uniceriate subulate hairs of 200–960 μm long, with single papillate basal cell and verrucose surface sculpturing. The distribution of stomata on the leaf surface is also similar in all three taxa. The stomata are paracytic, surrounded by (3) 5–7 epidermal cells, placed in a sunken position; they occur on the surface of both adaxial and abaxial sides, being more abundant in less haired plants.

By contrast, the distribution of hairs on the leaflet blade varies greatly, even within one species (Table S1,Fig. S1). The absence of hairs (on the adaxial surface in some individuals) and medium to dense hairiness (up to 160–222 hairs per mm^2) with trichome length (TL) varying from 150 to 812 μm were detected in *H. gmelinii* (Fig. S1, S3a and d). In *H. setigerum,* the hairiness density (TD) changed from medium (TDad = 24–100 hairs per mm^2) to dense (TDab = 100–244 hairs per mm^2), with trichome length ranging from 250 to 960 μm (Fig. S3b and e). *H. chaiyrakanicum* was significantly discriminated from *H. setigerum* based on its sparse hairiness density on adaxial (TDad) and abaxial (TDab) surfaces (13.7 ± 13.3 and 98.7 ± 52.5 trichomes per mm^2, respectively; Fig. S3c and f) and small trichome length (TLad = 331.0 ± 82.9 μm; TLab = 454.6 ± 101.9 μm). From *H. gmelinii* it differed only by its sparse hairiness density on the adaxial leaflet surface (TDad).

Our SEM findings indicated that hairs were mostly present on the leaflet margins, the abaxial surface and the midrib of all taxa, whereas on the adaxial surface they were less frequent, with the exception of several genotypes of *H. gmelinii* which possessed glabrous leaflets. Overall, the abaxial surface hairiness (TDab) was always denser and longer than the adaxial surface hairiness (TDab) in all studied *Hedysarum* taxa. The species most densely haired and with the longest trichomes on both adaxial and abaxial leaf surfaces was *H. setigerum.*

Analysis of the vegetative traits of the analyzed species revealed high coefficients of variation (CV) for most characteristics, ranging from 18–26 (number of leaflets, NL) to 63–120 (leaflet trichome density, TD), which indicates a prominent deviation in almost all vegetative traits. Most reproductive traits did not vary significantly at the intra-species level with the exception of the length of inflorescence (LI) which changed greatly in *H. gmelinii* (0.4–9.1 cm; CV = 60.4), *H. setigerum* (0.7–7 cm; CV = 54.1) and *H. chaiyrakanicum* (0.4–5.1 cm; CV = 48.9). The lowest deviation among reproductive traits was detected for the length of petals (e.g., the length of standard (LSD) varied from 1.1 mm in *H. chaiyrakanicum* to 1.5 in *H. setigerum,* with CV ranging from 9.4 to 11.7) and for the ratios between them (e.g., the K/S ratio among the studied species was 1; CV ranging between 6.1 and 9). Consequently, they can be considered as the most constant traits among the studied morphologic characteristics.

Regarding macromorphological traits, a significant correlation (+0.86) was detected between sprout (LS) and inflorescence lengths (LI) in *H. chaiyrakanicum* and the stem (LS) and the axis length (LA) in *H. gmelinii* (+0.71) and in *H. setigerum* (+0.84). There was a substantial positive correlation between the trichome densities on the adaxial (TDad) and abaxial (TDab) surfaces of the leaflet in *H. gmelinii* (+0.78). The trichome length (TL) slightly negatively correlated with the indumentum density (TD) on the adaxial (−0.59) and abaxial (−0.48) blade surfaces in *H. setigerum.*

The total amount of deviation of morphological characteristics found was the largest in *H. gmelinii* (306.0 for vegetative traits, and 199.3 for reproductive ones), and the lowest in *H. setigerum* (210.1 and 167.5, respectively).

The principal component analysis (PCA), based on morphological data revealed that the first three components with eigenvalues greater than 1 provided 92.0% of the total variation. PCA based on morphological data showed overlapped clusters of samples for the three studied species (Fig. 2a).

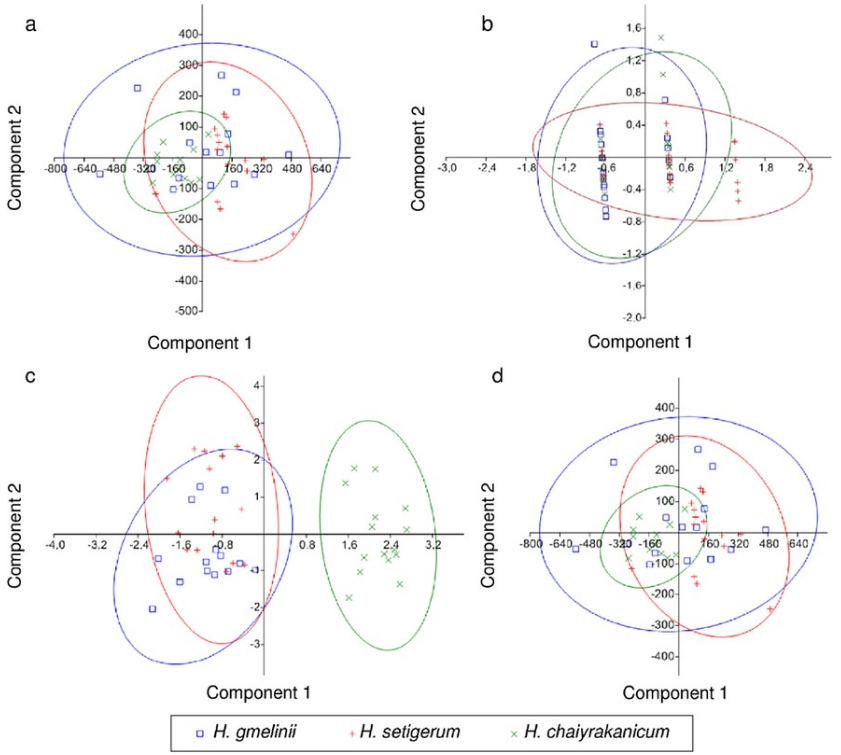

Fig. 2 Bidimensional PCA plots of the studied *H. gmelinii*, *H. setigerum* and *H. chaiyrakanicum* samples constructed with the first two canonical variables based on morphological (a), carpological (b), molecular ISSR (c) and combined (d) data.

alt-text: Fig. 2

3.2 Carpological variation

Our microscopy study indicated that no species-specific polymorphism in lomentum size, shape, and ornamentation could be observed among the three taxa (Table S1, Fig. S2). The study of lomenta in all species showed between two to six rounded segments united by a conspicuous articulation, ended in a small acute rostrum, and with obscure margins (Fig. S3g-i). The respective mature segments were 2.6–4.7 × 2.7–4 mm in size ($p > 0.05$); both segments had several lateral lines of spiny ribs often ending with more or less developed hooked prickles 430–880 μm long, with fluted cavernous surface, which were green in juvenile fruits and purple in mature ones (Fig. S3j). Occasionally, prickles were observed along the lomentum margin. Lomentum indumentum was dense, sericeous and presented unicellular flattened verrucose clavate trichomes 150–270 μm long and 55–60 μm wide, with hopper-shaped basis and a rounded, rarely acute, apex (Fig. S3k). In some *H. gmelinii* individuals, the trichomes were lanceolate, with tapering point. Our SEM study revealed the presence of lomentum trichomes with linear warts 1–3 μm long (Fig. S3l). The common cell type of lomentum surface for all studied *Hedysarum* taxa is 4-6-gonal, isodiametric to oblongate, 30–50 μm in diameter, having thick-walled cells with a rugose sculpture (Fig. S3m). The stomata were distributed sparsely (Fig. S3k). Based on scanning electron microscopy observations, the examined *Hedysarum* taxa showed little variation (CV ≤ 13.6) and did not differ significantly ($p > 0.05$) in quantitative pod segment size characteristics (segment length, SGL and width, SGW) (Table S1, Fig. S1).

The studied taxa were also similar to each other in having brown, flattened and reniform seeds, with protruded radical and small hilum. The mature seeds of *H. gmelinii* were on average 2.5 × 2.1 mm in size, the seeds of *H. setigerum* 2.3 × 2 mm, and those of *H. chaiyrakanicum* 2.5 × 2.1 mm. The variation in seed morphology was manifested mainly in a seed coat ornamentation (Fig. S3n-p). The surface of the most mature seeds of all species had a very similar irregular arrangement, showing a rugose-reticulate pattern. Polygonal elongate pits of outer deeply depressed periclinal walls were found on seed surface; anticlinal walls were thick and sinuous (type I, Fig. S3n). Occasionally, the seed coat surface was rugose and folded (type II, Fig. S3p). Some *H. setigerum* seeds presented irregular rugose-reticulate coat pattern with convex periclinal cell walls (type III; Fig. S3o). As one can see from Fig. 2b, PCA studies of carpological features did not found a gap for the delimitation of the species.

3.3 Molecular diversity

DNA quality estimated from the A_{260}/A_{280} ratio ranged from 1.71 to 2.02. Among 11 tested ISSR primers, 7 were successful in amplifying genomic DNA in *H. gmelinii*, *H. setigerum* and *H. chaiyrakanicum*, whereas four [$(CT)_8GC$, $(CA)_6A/GG$, $(GA)_6CC$, and $(CA)_6AC$] were omitted from the study because of the weak or unclear amplification pattern or due to the absence of PCR-products (Table 2).

Table 2 Genetic diversity parameters and PCR conditions of the ISSR primers used in the study. T_a, °C, annealing temperature; PB, number (percentage) of polymorphic bands; Rp, resolving power; H_0, Shannon' information index.

alt-text: Table 2

Primer	T_a, °C	PB (%)	Rp	H_0
$(CTC)_3GC$	42	23 (95.7)	7.5	7.3
$(CT)_8TG$	51	22 (100)	6.8	7.3
$(CA)_6GT$	47	26 (100)	12.5	11.0
$(CA)_6GG$	42	27 (100)	11.5	9.0
$(CA)_6AG$	47	20 (100)	6.5	6.8
$(CT)_8AC$	48	24 (100)	9.5	9.3
$(AC)_8C/TG$	58	25 (100)	8.4	8.5
Total	–	167 (99.4)	–	–

The ISSR markers were used to evaluate the 45 accessions (15 *H. gmelinii,* 15 *H. setigerum*, 15 *H. chaiyrakanicum*) under study. A total of 167 well-resolved bands showing a 99.4% percentage of polymorphism were obtained. The amplified fragment length ranged from 200 bp to 1.6 Kb in size. The number of amplified ISSR bands with each primer varied from 20 [$(CA)_6AG$] to 27 [$(CA)_6GG$]. Temperature of annealing ranged from 42 to 58 °C, depending on primer. The resolving power and the Shannon information index ranged from the greatest values of $(CA)_6GT$ (Rp = 12.5; H_0 = 11.0) to the lowest values of $(CA)_6AG$ (Rp = 6.5; H_0 = 6.8) (Table 2). Each individual accession showed a different ISSR profile from all others. Among 167 polymorphic ISSR fragments amplified, 27 bands were characteristic markers (species-specific), but not obligate for the species.

Nei' genetic distances between species pairs indicated that *H. gmelinii* and *H. setigerum* had the lowest genetic distance (D = 0.046), while *H. chaiyrakanicum* had the highest distances to its congeners, *H. gmelinii* and *H. setigerum* (D was 0.115 and 0.109, respectively) (Table 3).

Table 3 Pairwise Nei's genetic distances (D) based on ISSR polymorphism between studied species of the *H. gmelinii* group.

alt-text: Table 3

	H. gmelinii	*H. chaiyrakanicum*	*H. setigerum*
H. gmelinii	–	0.115	0.046
H. chaiyrakanicum	–	–	0.109
H. setigerum	–	–	–

PCA based on ISSR data revealed various levels of genetic differentiation among taxa (Fig. 2c). The cluster of *H. chaiyrakanicum* samples was the only group clearly separated from the two others along the first axis, which accumulated 13.9% of the variance, whereas the *H. gmelinii* and *H. setigerum* samples clustered together in the same bidimensional space. Similarly, the neighbor-joining tree identified two main genetic groups (Fig. 3). All accessions of *H. chaiyrakanicum* were nested in a divergent clade (A), whereas the individuals of *H. gmelinii* and *H. setigerum* were admixed in the same monophyletic clade (B). Within the *H. gmelinii* and *H. setigerum* group the individuals of *H. gmelinii* from northern Khakasia (pops. HG6, individuals 11–13, and HG8, individuals 24–27) joined in a subclade C (Fig. 3). The main clades recovered in the NJ tree coincide with the Structure output of K = 2 (Fig. S4) and support

two optimal genetic groups, which separate the *H. chaiyrakanicum* individuals from the individuals of *H. gmelinii* and *H. setigerum*. An AMOVA analysis showed that 13.9% of the total gene diversity was found among the natural populations, whereas the remaining 86.2% of the total variation occurred within the populations (Table S2).

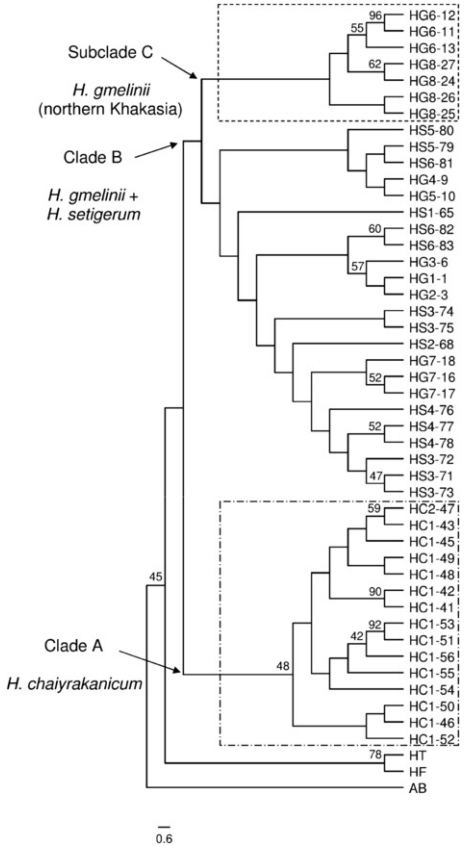

Fig. 3 Neighbor-joining tree based on Li & Nei genetic distances obtained from ISSR marker analysis from the studied *H. gmelinii*, *H. setigerum* and *H. chaiyrakanicum* samples. Outgroup samples of *Hedysarum theinum*, *H. fruticosum* and *Astragalus bifidus* were used to root the tree. Bootstrap support values greater than 40% are indicated on branches.

alt-text: Fig. 3

4 Discussion

4.1 Taxonomic value of morphological and carpological characters in the classification of the *H. gmelinii* complex taxa

To select the ~~of~~ useful morphological traits to separate close *Hedysarum* species is rather difficult and complicated by prominent pheno-morphological intra-species variation in habit, flower, and pod features. In one of the latest revision of the genus, Choi and Ohashi (2003) used floral, seed, pollen, and anatomical characteristics as diagnostic features to discriminate *H.* sect. *Spinosissima* as a distinct genus *Sulla* and to merge sections *Multicaulia*, *Crinifera* and *Subacaulia* into an enlarged *H.* section *Multicaulia*. The members of the southern Siberian *H. gmelinii* group have been traditionally separated from other *Hedysarum* taxa based on the stem length, number of leaflets, lomentum segments, color of flowers, lomentum surface features and geographic origin (Fedtschenko, 1972; Kurbatskij, 2006; Sa et al., 2010). Most of these traits have been analyzed in the current study. However, several morphological features that have been often treated as diagnostic characteristics are omitted from our investigation since stability of these traits depends on the maturity of the inflorescence and varies within species or populations, or even within a single plant. The neglected traits are the color of flower, length of inflorescence, number of flowers and lomenta per inflorescence, and number of segments per lomentum.

In a recent study, Sa et al. (2010) concluded that few phenotypic traits could separate *H. gmelinii* from its close congeners *H. setigerum* and *H. dahuricum*; the authors proposed the stem length and color of flowers as the most diagnostically effective traits to differentiate them. However, our observations have demonstrated the high plasticity of both traits, making them useless to resolve the taxonomy of the *H. gmelinii* group. In *Hedysarum*, color of flower is often a highly variable character. For instance, we observed plants with both lilac and white flowers within a single population of *H. gmelinii* (Khakasia, HG6). A change in the color of the flower is also typical of another *Hedysarum* species, *H. astragaloides*, which shows young pink or red flowers turning yellowish white after anthesis (Lal et al., 2014), and of *H. garinense*, which has pale yellow flowers at first, becoming purple-violet later (Dehshiri et al., 2012). The wide phenotypic variability in the color of flower along with a prominent overlapping of the stem length in different species precluded the use of this feature as a powerful taxonomic marker for the *H. gmelinii* complex taxa.

The presence (or absence) and abundance of the rachises produced by the last year's sprouts surrounding the basis of the stem (R) were measured in the studied *Hedysarum* species for the first time. A higher density of rachises in *H. chaiyrakanicum* (Table S1) supports this trait as a diagnostic feature for resolving the taxonomy of short-stemmed *Hedysarum* species.

The biological implication of these observations could be connected with the physical conditions of the habitat of each species. The Central Tuva environment where *H. chaiyrakanicum* grows is characterized by dry intermontane depressions that have remarkable cold winter climate (~~http://www.rusnature.info/reg/14_4.htm~~Russian Nature, 2016). In the Tuva depression, the average January temperature is –34 °C (down to the absolute minimum of –58 °C) and the average July temperature is 16 °C, therefore the amplitude of annual absolute temperatures can reach 80°C. The average frost-free period lasts only 120 days. Our morphological data might reflect the adaptiveness of the species to this harsh climate. *H. chaiyrakanicum* shows biological features that favor its survival in this environment like a negligible rosette stem and dense covering of rachises which protect the wintering buds. Longer sprout vs less vegetative biomass (smaller number of leaflets) compensate for the short stem and facilitate the production and effective dispersal of seeds during a short reproductive season.

Micromorphological traits play an important role in the plant taxonomy (Metcalfe and Chalk, 1957). The leaf, petal, and fruit micromorphology provide the important characteristics in separation the taxa at different hierarchic levels in the Fabaceae family (Ghahremani-Nejad, 2004; Shaheen, 2008; Noori et al., 2014). In *Hedysarum*, the leaf hairiness, fruit, flower and pollen morphology, and seed coat ornamentation are of taxonomic value (Choi and Ohashi, 2003; Ghanavati and Amirabadizadeh, 2012; Dural and Citak, 2015) and have been used for the identification and description of new species (Sa, 2007).

In our study, the hair type, length and distribution were investigated both on adaxial and abaxial leaf blade surfaces in the three species of the *H. gmelinii* group. We found that the indumentum of leaflets was close to that of *Dichilus, Argyrolobium* and of some *Polhilla* species (Fabaceae), characterized by the presence of non-glandular verrucose hairs (Moteetee et al., 2002). Statistically significant ($p < 0.05$) values of leaflet hairiness separated *H. gmelinii* and *H. setigerum* from *H. chaiyrakanicum*, whereas they could not discriminate among the former species. The leaflets of *H. chaiyrakanicum* showed significantly less hair density and hair length than those of *H. setigerum* ($p < 0.05$). By contrast, in *H. gmelinii,* the hair length and distribution varied from the absence of hairs to the densely haired pattern in different individuals, which showed the highest variability among all examined features (CV = 120). It can be concluded that this trait could not be used to characterize the later species.

In addition to the gross pheno-morphological evidence, the distribution pattern of palynological and carpological features have been shown to be useful in improving infrageneric classification in *Hedysarum* (Mironov, 2000; Sa et al., 2010; Ranjbar et al., 2008; Ghanavati and Amirabadizadeh, 2012; Dural and Citak, 2015) and related genera *Onobrychis* (Noori et al., 2014) and *Desmodium* (Shaheen, 2008). However, the results of LM analyses of lomentum found no significant differences between the studied species. All taxa showed a similar size, shape and surface features for the mature lomentum (see Fig. S2), which is usually densely haired and armed with hooked prickles. A closer inspection of the lomentum SEM traits, such as the epidermal cell pattern and indumentum, and of seed size also failed to detect any interspecific difference. Furthermore, the inspection of lomentum indumentum shows the linear warts covered with trichomes (Fig. S3l) as in *H. pannosum,* a less related species from sect. *Crinifera* (Boiss.) B. Fedtsch. (Dural and Citak, 2015).

The seed ornamentation data provide a valuable source of discriminant traits at different hierarchical levels in angiosperms, but not in all cases. For example, a significant variability of seed types was found in two controversial species of *Trollius* L., though the seed sculpture characteristics could not be used in the species discrimination (Antkowiak et al., 2010). Similarly, 7 types of seed morphology were recognized within 37 taxa of *Trigonella* L. (Ceter et al., 2012), however, seed characteristics did not provide any considerable information to separate the species even at sectional level.

Previous SEM studies detected two types of seed coat ornamentation within *Hedysarum*, concave rugose-reticulate (type I) or irregularly folded (type II) coats (Sa, 2007; Sa et al., 2010; Dural and Citak, 2015). Interestingly, we have found the third type of seed coat that has not been recorded before for *Hedysarum*. Some *H. setigerum* samples showed a convex and reticulate seed ornamentation with depressed sinuate periclinal cell walls (type III) (Fig. S3o). Although this synapomorphy (convex seed micro-ornamentation) is unique in the *H. gmelinii* group, it is only present in some *H. setigerum* accessions and could not discriminate this species from its congeners. Our analysis shows that most individuals of *H. gmelinii, H. setigerum* and *H. chaiyrakanicum* have a prevalent and homomorphic seed coat ornamentation of type I, precluding the use of this characteristics to differentiate them. Consequently, the carpological features have limited application in the taxonomy of the *H. gmelinii* group species.

4.2 Genetic ISSR markers support phenotypic differences and the separation of *H. chaiyrakanicum* from *H. gmelinii* sensu lato (incl. *H. setigerum*)

Hypervariable genetic markers such as ISSR, which generate a high number of repeatable polymorphisms, have been widely used in the taxonomic studies of plants, as well as in germplasm evaluation and plant conservation programs (Gilbert et al., 1999; Rao et al., 2007; Abraham et al., 2015). The high fragment polymorphism and fidelity of reproduction of ISSR patterns make ISSR markers a helpful tool in investigating the taxonomy of complex plant groups at the species and interspecies levels. However, information on the application of ISSR markers in ~~temperate northern Asian~~ *Hedysarum* species was insufficient and mostly aimed to detect variability and differentiation in crop species *H. coronarium* and its cultivars (Ruisi et al., 2011; Marghali et al., 2012). Various molecular DNA markers including ISSR were studied to resolve phylogenetic relationships among Mediterranean *Hedysarum* and *Sulla* species (Chennaoui-Kourda et al., 2007; Marghali et al., 2014).

Our genetic study based on ISSR markers support the genetic divergence of *H. chaiyrakanicum* from its congeners *H. gmelinii* and *H. setigerum*, as indicated in the PCA (Fig. 2c), neighbor-joining (Fig. 3) and Structure (Fig. S4) analyses. The ISSR profiles reconstruct the genetic entity of this endemic species, comprised of two isolated populations from the Central Tuva (HC1 and HC2; Fig. 1). The *H. chaiyrakanicum* clade includes the accessions collected from northern, north-eastern, and western slopes of the Khairakan mountain (pop. CH1, locus classicus) and individuals collected from the outskirts of the Semiozersk village (pop. CH2) located at approximately 105 km distance from Khairakan and separated from it by the Kurtushibinsky mountain ridge.

By contrast, the genetic data does not support the separation of *H. setigerum* from *H. gmelinii*. Therefore, all the conducted analyses show an overlap or admixture of individuals from the two species (Figs. 2 and 3) and classification within the same genetic group (Fig. S4). The hierarchical AMOVA tests also select two groups (*H. chaiyrakanicum* vs *H. gmelinii* and *H. setigerum*) as the best genetic partition (Table S2) and indicate that the genetic differentiation is found mainly within populations. The monophyletic origin of *H. gmelinii* accessions sampled from two Khakasian populations (clade C, Fig. 3) could be explained by the closest geographic distance (about 15 km, see Fig. 1) between them, which does not preclude the pollinators' migration, facilitating the genetic drift and therefore it may prevent population diversification.

The close genetic relatedness obtained for the *H. setigerum* and *H. gmelinii* samples agrees with their similarities in morphological and carpological features, indicating that all examined morphological and molecular characteristics do not provide any considerable information that could be used to distinguish them. Consequently, we have found no reliable evidences that would reinforce the status of *H. setigerum* as a separate species, and therefore propose to subsume it within *H. gmelinii*. Comparisons of the geographic ranges of these two species suggest that *H. setigerum* may be considered as a short-stemmed ecotype of *H. gmelinii* located in southern Siberia as it was proposed by Fedtschenko (1902) and further by Kurbatskij (2006) who classified it as *H. gmelinii* subsp. ~~S~~*s**etigerum* (Turcz. ex Fischer et Meyer) Kurbatsky. Genetic polymorphism, large amount of variation in chromosome numbers and polyploidy (Yan et al., 1989; Kurbatskij, 2006~~; Yan et al., 1989~~) might have driven the phenotypic diversification and geographic distribution of *H. gmelinii* (Doyle, 2012), making difficult the taxonomic delimitation of related taxa. Nonetheless, a larger survey involving all the short-stemmed *Hedysarum* species from the *Multicaulia* and *Subacaulia* sections would be necessary to clarify the taxonomy of northern Asian legumes.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bse.2016.10.001.

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Appendix A. Supplementary data

The following is the supplementary data related to this article:

Multimedia Component 1

Online material

alt-text: Online material

Highlights

- The *H. chaiyrakanicum* is well discriminated by its ISSR and morphological profiles.
- Our study provided no evidences for the separation of *H. gmelinii* and *H. setigerum*.
- *H. setigerum* should be treated as a subspecies of *H. gmelinii*.
- Based on the Nei' standard coefficients, the species of *H. gmelinii* group have evolved recently.
- The ISSR profile is found to be an important diagnostic feature in biosystematics of *Hedysarum*.

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