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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*.

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

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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).