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Resolving the position of *Astragalus borysthenicus* Klokov within the *Astragalus* L. species

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Abstract: The present study is focused on several species from the genus *Astragalus* L. with the aim to clarify the taxonomic status of Ukrainian local endemic species *Astragalus borysthenicus* Klokov, which is sometimes considered a synonym to *A. onobrychis* L. In this study, the morphological features, current taxonomy, taxonomical history, and phylogenetic analysis based on rDNA Bayesian inference, as well as comparative analysis of ITS1 and ITS2 secondary structures, were investigated. It was found that *A. borysthenicus* is distant from *A. onobrychis* according to phylogenetic analysis. Moreover, *A. borysthenicus* has differences from the investigated taxa in its secondary structures of ITS1 and ITS2 transcripts. These data suggest that *A. borysthenicus* should be treated as a separate species rather than a synonym to *A. onobrychis*.

Key words: *Astragalus*, Bayesian inference, ITS secondary structure, phylogeny, taxonomy

1. Introduction

Astragalus L. (Fabaceae) can be considered one of the largest genera of flowering plants. The exact number of species remains unknown and varies from about 2500 (Ekici et al., 2015) to 3000 species (Hardion et al., 2010) that are mainly distributed in the northern hemisphere. Many *Astragalus* species are European only (Hardion et al., 2010).

The flora of vascular plants of Ukraine includes 5187 species and infraspecific taxa from 160 families, among them 380 species of the family Fabaceae. Fifty-three species belong to *Astragalus* (Mosyakin and Fedoronchuk, 1999), 15 of which are endemic (28.3%). In comparison, the endemism rates of *Astragalus* in different geographical regions of East Europe and the Caucasus are as follows: East European plain – 55.2%, Crimea – 44.8%, Urals – 56.8%, Pre-Caucasus – 17.6%, Caucasus – 64.5%, Transcaucasia – 65.7% (Sytin, 2009). A total of 425 *Astragalus* species are listed for Turkey, and their endemism rate is about 51% (Podlech and Zarre, 2013; Ekici et al., 2015).

Astragalus borysthenicus Klokov is a Ukrainian local endemic species listed in the Red Data Book of Ukraine (Krytska, 2009), the European Red List of Globally Threatened Animals and Plants (United Nations, 1991), and regional red lists (Korzhenevskiy et al., 2012). It ranges across the littoral zones of the Black Sea and the Sea of Azov, but the populations are damaged by recreational and commercial construction, as well as military conflicts on the coast. It occurs mostly solitary or in small groups; sometimes it is the predominant component of plant communities (*A. borysthenicus* coverage varies from solitary individuals up to 65%). Under anthropogenic influences, populations are transformed into insignificant localities with a constantly decreasing number of individuals. Complete populations that include plants at different stages of growth are distributed mostly in protected areas of the coast of the Sea of Azov (total area is approximately 15 ha). The density of these populations is 1–3 individuals/m^2; mature individuals prevail (Korzhenevskiy et al., 2012).

The taxonomic status of *A. borysthenicus* is interpreted ambiguously and remains elusive in various scientific studies. Sometimes it is considered a separate species (Visyulina, 1954; Vasilieva, 1987; United Nations, 1991; Mosyakin and Fedoronchuk, 1999). However, in many other taxonomic and floristic publications it is cited as a

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junior synonym of nonthreatened multiregional (Europe–Asia–Africa) *Astragalus onobrychis* L. (Borisova, 1946; Chater, 1968; Cherepanov, 1995; Ekici et al., 2015). Both taxa belong to sect. *Onobrychoidei* DC., whose main diagnostic characteristic is pubescence with mostly medifixed or asymmetrically bifurcate hairs (Podlech and Zarre, 2013). Ambiguity in the taxonomical status of *A. borysthenicus* may cause the loss of its endemic and protected status.

When morphological characteristics are not enough, molecular analysis can help resolve the problem with separation of close taxa. Nuclear ribosomal spacers (ITS1 and ITS2) are widely used as molecular phylogenetic markers for plants because of their universality, simplicity in amplification, intragenomic uniformity, and variability at the specific, generic, and family levels (Baldwin et al., 1995; Álvarez and Wendel, 2003). Moreover, ITS2 secondary structure analysis allows to assess the level of reproductive isolation and to distinguish taxa (Coleman and Mai, 1997; Coleman, 2000, 2007; Ruhl et al., 2009).

ITS sequences obtained without cloning may contain ambiguous sites. This can happen due to PCR and sequencing errors (Clarke, 2001; Kunin et al., 2011), or can be the signature of intragenomic polymorphism. Considering that from 30% to 70% of all flowering plant species had hybridization events in their phylogenetic histories (Wendt et al., 2001), another reason for the presence of ambiguous sites is hybridization (Hřibová et al., 2011). However, a number of plant sequences have no ambiguous positions and it is reasonable to suggest that they most likely did not have hybridization events in the past. Therefore, such organisms can probably be treated as relative analogs of "pure homozygous lines" or true breeding organisms (TBOs) (King et al., 2007).

Thus, the aim of our study was to assess the taxonomical status of *A. borysthenicus* using morphological feature comparisons, phylogenetic reconstructions, and analyses of secondary structures of ITS1 and ITS2 transcripts. Moreover, an additional aim was to find a "pure" nonhybrid *A. borysthenicus* (TBO) or to ascertain the probability of the hybridogenic origin of analyzed specimens of *A. borysthenicus*. We also used the same type of data for genetically related species (*A. dasycarpus* Chamberlain, *A. akmanii* Aytaç & H.Duman, *A. ansinii* Uzun, Terzioğlu & Pal.-Uzun, *A. viridissimus* Freyn & Sint., and *A. bachmarensis* Grossh.) to assess the level of separation of *A. borysthenicus*.

2. Materials and methods

2.1. Morphological analysis

Morphological features of *A. borysthenicus* and *A. onobrychis* were analyzed in detail according to their protologues and other special sources (Linnaeus, 1753; Bunge, 1868; Grossheim, 1930; Borisova, 1946; Klokov, 1946; Visyulina, 1954; Vasilieva, 1987; Ekici et al., 2015) in order to create a list of morphological characters that potentially differentiate the studied taxa. A comparative list of *A. borysthenicus* and *A. onobrychis* morphological characters was completed on the basis of mentioned protologues and other special sources, field experience, and the study of herbarium material from KW and KWU (approximately 100 specimens).

Morphological analysis of the *Astragalus* species from Turkey and adjacent areas (*A. bachmarensis*, *A. akmanii*, *A. dasycarpus*, *A. ansinii*, and *A. viridissimus*) was also used to verify the presence of morphologically similar parameters between *A. borysthenicus* and related species endemic to Turkey or the Western Caucasus. Morphological features were analyzed according to protologues and other special sources (Freyn, 1892; Grossheim, 1930; Chamberlain and Matthews, 1969, 1970; Duman et al., 1995; Güner et al., 2000; Uzun et al., 2009). Only those characteristics that were comprehensively represented in the available literature for all comparable species were taken in order to create a list of morphological characters that potentially differentiate these taxa. The names of *Astragalus* sections are given according to Podlech and Zarre (2013).

2.2. Molecular analysis

2.2.1. Plant material

Three herbarium specimens of *A. borysthenicus* and two specimens of *A. onobrychis* were used for molecular analysis. Data on vouchers and origins of plants are presented in Table 1.

2.2.2. DNA extraction, amplification, sequencing, and annotation

Total genomic DNA was extracted from herbarium material using a modification (Tarieiev et al., 2011) of the method developed by Doyle and Doyle (1990). PCR reactions of ITS1-5.8S-ITS2 regions were performed as described by White (1990) using ITS1 and ITS4 universal primers. Sequencing was carried out by Macrogen Inc. and obtained sequences were deposited in GenBank (see Table 1).

Sequences were assembled and edited manually using BioEdit software (Hall, 1999). Ambiguous positions were coded using the NC-IUPAC ambiguity codes if two peaks were present at the same position and the lower peak was more than 1/2 of the higher peak in the chromatograms. Additionally, we analyzed the presence of ambiguous positions in sequences of *A. bachmarensis* (JQ685625), *A. akmanii* (JQ685622), and *A. dasycarpus* (JQ685634) from previous investigations (Dizkirici et al., 2013) whose ITS sequences are similar to *A. borysthenicus* and *A. onobrychis*.

Table 1. Voucher data and GenBank accession numbers for specimens used in this study.

No.	Taxon name	GenBank accession number	Herbarium acronym and specimen identifier	Location, date, collector
1	*A. borysthenicus*	KY973970	KW 022320, neotype	Ukraine, Kherson reg., Genichesk distr., Birjuchiy Island, 20.07.1953, M. Parnasky
2		KY973969	KWU 004461	Ukraine, Kherson reg., Genichesk distr., southern part of Fedotova maritime spit, sand-shell steppe, 13.07.1999, O. Tyshchenko
3		KY973968	KW	Ukraine, Crimea, Feodosia distr., near the Beregove village, 21.05.1974, O. Dubovyk
4	*A. onobrychis*	KY973971	KW 001968	Ukraine, Donetsk reg., Amvrosievka distr., Kalinove village, Shiroka ravine, 17.06.1986, L. Krytska
5		KY973972	KWU 058918	Ukraine, Kirovograd reg., Bobrynets, steppe, 26.07.2009, O. Kovalenko

The 5'-end of ITS1 sequences was annotated by folding in mFOLD (Zuker, 2003) so that its structure corresponds to the model of Boraginales (Gottschling et al., 2001). The 3'-end of ITS1 was annotated according to *A. bachmarensis* (JQ685625) from GenBank. Annotation of ITS2 region was performed according to the concept of finding 5.8S-26S complementary fragments that form a hybrid stem (Gottschling and Plötner, 2004) using mFOLD (Zuker, 2003).

2.2.3. Phylogenetic analysis

Two nrDNA sequences of *A. borysthenicus* and two of *A. onobrychis* (sect. *Onobrychoidei*) were used to infer their phylogeny. A part of the sampling was taken from the ones most similar to *A. borysthenicus* and *A. onobrychis* ITS sequences according to the results of a BLAST search (*A. viridissimus* FJ613404, *A. ansinii* FJ613403, *A. bachmarensis* JQ685625, *A. akmanii* JQ685622 from sect. *Hypoglottidei* DC., *A. dasycarpus* JQ685634 from *Brachylobium* Boiss.). Other sequences obtained from GenBank were chosen to represent taxa from the sections listed above. Finally, 21 nrDNA sequences of *Astragalus* were used to build a phylogeny. Two sequences of *Oxytropis* DC. were used as an outgroup. The list of imported sequences comprised *O. pilosa* (L.) DC. KM053396, *O. pallasii* Pers. KM053395, *A. viciaefolius* DC. JQ685637, *A. sevangensis* Grossh. AB727521, *A. aduncus* Willd. KX954888, *A. bijarensis* Podlech & Sytin AB727510, *A. xerophilus* Ledeb. AB727526, *A. lasioglottis* M.Bieb. JQ685644, *A. vexillaris* Boiss. JQ685663, *A. tibetanus* Bunge KX955039, *A. zerdanus* Boiss. AB051964, *A. penetratus* Maassoumi KX955003, *A. abditus* Podlech KX954886, *A. daenensis* Boiss. KX9549230, *A. viridissimus* FJ613404, *A. ansinii* FJ613403, *A. bachmarensis* JQ685625, *A. akmanii* JQ685622, and *A. dasycarpus* JQ685634.

All sequences were aligned using ClustalW (Thompson et al., 1994) and then converted to Nexus format. The optimal evolutionary model was selected in Modeltest 3.7 (Posada and Crandall, 1998). The phylogenetic tree was constructed using the MrBayes 3.2 program package (Ronquist et al., 2012). The GTR+I+G model (Tavaré, 1986) and 1 million iterations for analysis were used.

2.2.4. ITS1 and ITS2 secondary structures

Models of ITS1 and ITS2 transcripts were built in mFOLD (Zuker, 2003) by sequential folding of helices. ITS1 and ITS2 secondary structures were determined according to previously published models (Coleman and Mai, 1997; Gottschling et al., 2001; Coleman, 2007). The obtained models were visualized by Pseudoviewer 3.0 (Byun and Han, 2009). The dataset for ITS1-ITS2 secondary structure comparison is composed of sequences that are the most similar to *A. borysthenicus* and *A. onobrychis* according to BLAST (Altschul et al., 1990): *A. viridissimus* FJ613404, *A. ansinii* FJ613403, *A. bachmarensis* JQ685625, *A. akmanii* JQ685622, and *A. dasycarpus* JQ685634.

3. Results

3.1. Morphological analysis

Comparative morphological data analysis of *A. borysthenicus* and *A. onobrychis* (Table 2) using the data published in initial descriptions showed that it does not seem possible to determine clear hiatuses in morphological

Table 2. Comparative characteristics of *A. onobrychis* and *A. borysthenicus*.

Type of taxonomic characters	*A. onobrychis*	*A. borysthenicus*
Morphological features with clear hiatuses	Whole plant is haired with gray adpressed hairs	Stems ciliate with black and white hairs
	Bracts shorter than calyces	Bracts of the same length as calyces
	Corolla pink-lilac, lilac-purple, purple, 1.5–2.3 cm long	Corolla dark blue-purple, 2.5–3.0 cm long
	Legumes 0.7–1.1 cm long, with nearly straight neck	Legumes 1.4–1.5 cm long, with slightly curved neck
Morphological features with unclear hiatuses	Stem (6–)20–80 cm high	Stem 50–70 cm high
	Leaf length 5–10 cm, 15–31 leaflets	Leaf length 10–15 cm, 10–17 pairs of leaflets
	Leaflets oblong, oblong-linear, narrowly elliptic	Leaflets oblong-elliptic, oblong-lanceolate, or linear
	Inflorescence longer than leaves	Inflorescence two times longer than leaves
	Racemes dense, capitate or elongated, spiciform, to 10 cm long when fruiting	Capitulum oval, dense and elongated to the end of flowering
	Calyx with short tube and teeth of equal length to 2–3 times longer than the tube	Calyx tubular-funnel with teeth of equal length to the tube
	Standard length 2.0–2.5 cm	Standard length 2.5–3.0 cm
	Flowering period VI–VII	Flowering period V–VII
	Habitats at steppes, slopes, outcrops, sands; whole habitat - Middle, Atlantic and East Europe, Balkans, Caucasus, Western Siberia and Asia Minor	Habitats at coastal and river sands, only at the far south of Ukraine
Morphological features without hiatuses	Stems erect or ascending	
	Leaflets length 1–2.5 cm	
	Stipules ovate, joined at base, with a pointed top	

features between these taxa. The protologue of *A. onobrychis* cannot be used for comprehensive comparisons since there is no detailed description of the species. Linnaeus (1753) mentioned only a few parameters: herbaceous perennial plant with branchy stems, spiciform inflorescence, double-veined vexillum, purple flowers, habitat in Austria (the citation of *A. onobrychis* in the initial description by Linnaeus (1753): "... caulescens diffusus, pedunculis spicatis, vexillis flore duplo longioribus. Onobrychis spicata, flore purpureo..."). None of the mentioned parameters are accompanied by numerical data. More detailed data about the morphological characters of *A. onobrychis* appeared

in specialized literature published much later (Bunge, 1868; Grossheim, 1930; Borisova, 1946; Ekici et al., 2015). However, there are a few clear hiatuses observed from the following features of *A. onobrychi*s and *A. borysthenicu*s. Important taxonomic characters with unclear hiatuses and without hiatuses are also given in Table 2.

According to morphological data analysis (Table 3), investigated species from Turkey and adjacent areas (*A. dasycarpus*, *A. bachmarensis*, *A. akmanii*, *A. ansinii*, and *A. viridissimus*) in most cases have unclear hiatuses but clearly differ from *A. borysthenicus*.

3.2. Sequence analyses

ITS1-5.8S-ITS2 sequences of *A. borysthenicus* are slightly different. Their similarity ranges from 98.92% to 99.63%. Both sequences of *A. onobrychis* are identical and differ only in sequence length. The sequence of *A. borysthenicus* KY973968 contains both full ITS1 and ITS2 regions; thus, it was used for a BLAST search of similar taxa. The sequence of *A. onobrychis* from the Donetsk region was used in the same way.

According to the results of the BLAST search, the sequence of *A. borysthenicus* (KY973968) is most similar to *A. viridissimus* (FJ613404) and *A. ansinii* (FJ613403) (97.84% identity, 100% coverage). The sequence of *A. onobrychis* (KY973971) is most similar to *A. bachmarensis* (JQ685625) – 99.67%, *A. akmanii* (JQ685622) – 99.51%, and *A. dasycarpus* (JQ685634) – 99.18%.

Both sequences of *A. onobrychis* do not have any ambiguous positions and therefore can be treated as TBO. On the other hand, sequences of *A. borysthenicus* are different: the specimen from Crimea (KY973968) does not have any ambiguous positions and seems to be "pure" *A. borysthenicus*, while the specimens from the Kherson region have ambiguous positions (KY973969 – two, KY973970 – six).

The sequences of *A. bachmarensis* (JQ685625), *A. akmanii* (JQ685622), and *A. dasycarpus* (JQ685634) were also analyzed. These sequences do not have any ambiguous sites and therefore represent "pure" taxa.

3.3. Phylogenetic analysis

The alignment of 23 nrDNA ITS sequences (including two outgroups) produced a matrix of 543 bp in length. This dataset contains 83 (15.29%) variable sites.

Phylogenetic relationships of *A. borysthenicus*, *A. onobrychis*, and other *Astragalus* species are presented in Figure 1. *A. borysthenicus* forms a separate clade, sister to *A. aduncus* and *A. sevangensis*, and clearly distant from *A. onobrychis*. *A. borysthenicus* is distant from similar taxa as well according to the BLAST search – *A. viridissimus* FJ613404 and *A. ansinii* FJ613403.

Phylogenetic relationships between sections are poorly resolved. Taxa that belong to sect. *Onobrychoidei* form a single clade, except for *A. bachmarensis*, which belongs to *Hypoglottidei*. Most taxa from sect. *Brachylobium* form a clade with strong support, as well, but *A. dasycarpus* is positioned within taxa from *Hypoglottidei*. Sect. *Hypoglottidei* itself seems to be paraphyletic according to this phylogenetic analysis.

3.4. ITS1 secondary structure comparison

The model of ITS1 secondary structure of *A. borysthenicus* consists of four main (helix 1–4) and two additional (a and b) helices (Figure 2). The ITS1 secondary structures of other investigated taxa are similar to *A. borysthenicus* but have differences in 11 sites; four of them are hemi-compensatory base changes (hCBCs) (Table 4).

The most distant specimen is *A. onobrychis* from Ukraine: it differs in two hCBCs (81.A→G, 191.C→U) from all other sequences in the dataset. *A. viridissimus* (FJ613404) and *A. ansinii* (FJ613403) also differ from other sequences in one hCBC in the 74th site (U→C) and three nonstructural substitutions (nst) – C→U in the 27th site, U→A in the 76th, and U→C in the 225th. *A. borysthenicus* sequences differ from others by one hCBC (183.U→C). Other taxa are more similar. *A. dasycarpus* differs from *A. bachmarensis* and *A. akmanii* only in two nst, while the ITS1 secondary structures of the last two mentioned species are identical. The sequence of the *A. borysthenicus* neotype (KY973970) has four ambiguous nucleotides in the 75th (Y), 94th (Y), 174th (M), and 183rd (Y) sites. *A. borysthenicus* KY973969 has one ambiguous nucleotide (Y in 94th).

3.5. ITS2 secondary structure comparison

The ITS2 secondary structures of investigated taxa consist of four helices. They are similar to *A. borysthenicus* (Figure 3) but have differences in nine sites. Among them, 6 differences are more important (two structural substitutions and 4 hCBC) (see Table 4).

The ITS2 secondary structures of *A. borysthenicus* KY973969 and KY973970 differ from other investigated taxa in hCBCs (10.U→C, 168.G→A), structural substitution (sst) (101.A→C), and nst (172.A→C, A→U). The neotype specimen of *A. borysthenicus* (KY973970) has two ambiguously identified nucleotides in the 10th and 101st sites – Y and M, respectively. *A. viridissimus* and *A. ansinii* differ from other sequences in the dataset in two hCBCs (146.A→G, 203.A→G) and two nst (36.A→C, 95.C→U). *A. dasycarpus* and *A. akmanii* differ from *A. bachmarensis* in one nst (172nd site).

4. Discussion

The results obtained from ITS2 secondary structural comparison of the investigated taxa have shown the absence

Table 3. Comparative characteristics of *A. borysthenicus* and related species from Turkey and adjacent areas.

Character	*A. borysthenicus*	*A. dasycarpus*	*A. bachmarensis*	*A. akmanii*	*A. ansinii*	*A. viridissimus*
Section	*Onobrychoidei*	*Brachylobium*	*Hypoglottidei*			
Stems	Ascending (erect or ascending), more or less numerous, 50–70 (50–80) cm	Dwarf, slightly raised up to circa 5 cm	10–20 (10–30) cm, usually prostrate	Prostrate to erect, rhizomatous, herbaceous, 3–10 cm, simple or branched	Prostrate, 10–20 cm long, 1–2 mm thick	Prostrate to erect, dwarf, herbaceous, numerous, 5–15 cm
Leaflet pairs	10–17	6–10	5–9 (6–9)	7–13	7–13	4–7 (6–10)
Stipules	With a broad-ovoid base, adnate, apex more or less elongated	Triangular, adnate at the base	Lanceolate to triangular, adnate for at least half of their length	Triangular-lanceolate, long-acuminate, adnate at the base	From oblong-triangular to narrowly triangular, acuminate, membranaceous, reddish or greenish, adnate for at least half of their length	Triangular-lanceolate at the apex, herbaceous or as the exception membranaceous, adnate at the base
Bracts	Linear-lanceolate, equal with calyx tube	Circa 4 mm, lanceolate	Circa 5 mm, linear-lanceolate to lanceolate (acute-lanceolate), one-third shorter than a calyx tube	4–6 mm, linear	Circa 3 mm long, lanceolate	Oval-lanceolate, with filmy edge, circa 3 mm long
Peduncles	2 times longer than leaves	2–3 cm	1.5–2 times longer than leaves	3–8 cm	2.0–3.5 cm long, glabrous, shorter than leaves	3–5 cm
Inflorescence	Racemes (capitula) short, oval, dense, at the end of flowering elongated	Globose, 4–8-flowered spike	Oval, dense, subcapitate, 7–12-flowered spike, during fruiting does not elongate	Ovate to capitate, becoming cylindrical in fruit, 15–50-flowered	Subcapitate, 3–8-flowered spike	Racemes sparsely flowered, slightly capitate at the apex
Calyx	Tubular-funnel, 8–11 mm (up to 1 cm), with linear-awl teeth, teeth are equal/almost equal to the tube, with short black and white hairs	8–10 mm, tubular, densely pubescent with numerous black hairs and a few white spreading hairs, teeth circa 2 mm, linear	Circa 9 mm, cylindrical (tubular), almost not inflated, densely spreading simple-hairy (with black and white hairs), teeth circa 3 mm (teeth three times shorter than a tube)	Densely pubescent with long white hairs, 9–12 mm, teeth 4–8 mm, linear	5–6 mm, tubular, the whole surface glabrous, splitting at fruiting time, teeth narrowly triangular, acute to acuminate, 2–3 mm	Sparsely pubescent with deflected white pellucid and black hairs, sepals lanceolate, 4 of them form tube 10 (7–10) mm long, with shortened edges, teeth 2–2.5 mm, sparsely pubescent with short simple hairs
Corolla	Dark blue-violet (dark blue-purple, dark blue or dark violet)	Lilac	Violet (bright violet-blue, bright violet)	White to cream	Glabrous, lilac beyond the pale claws, violet-purple when dry	Bluish when dry

Table 3. (Continued).

Standard	25–30 × 7–8 mm	18–22 mm	19–21 mm (1.5 times longer than wings)	9–11 mm (6–7 × 4–5 mm)	20–22 mm	16–18 mm (2 times longer than calyx)
Legume size and indumentum	14–15 × 4 mm, densely pubescent with quite long white distant-hairs, beak circa 2 mm, slightly curved	Circa 10 mm, ovoid, laterally compressed, densely pilose with long white spreading hairs, beak circa 2 mm	Circa 12 mm (18–20 × 4–5 mm), oblong with profuse, spreading, black hairs and sparse, longer stiff white hairs (irregularly granular, densely hairy, in the lower part often densely pubescent with white hairs, top is suddenly narrowed to a straight beak circa 4 mm)	5–19 × 8–10 mm, ovoid, inflated, white pilose, beak 4–6 mm, slightly curved, pilose	13–14 × 4 mm, erect to spreading with a linear 1–2 mm long stipe, bilocular, ovoid, carinate ventrally, widely and deeply grooved dorsally, glabrous, with beak 2–3 mm long, straight to gently curved downwards	(Ripe) erect-inclined, sparsely pubescent with compressed white hairs, oblique-elongated, slightly curved, legume length is three times smaller its width, with acute beak 3–17 × 5–5.5 mm

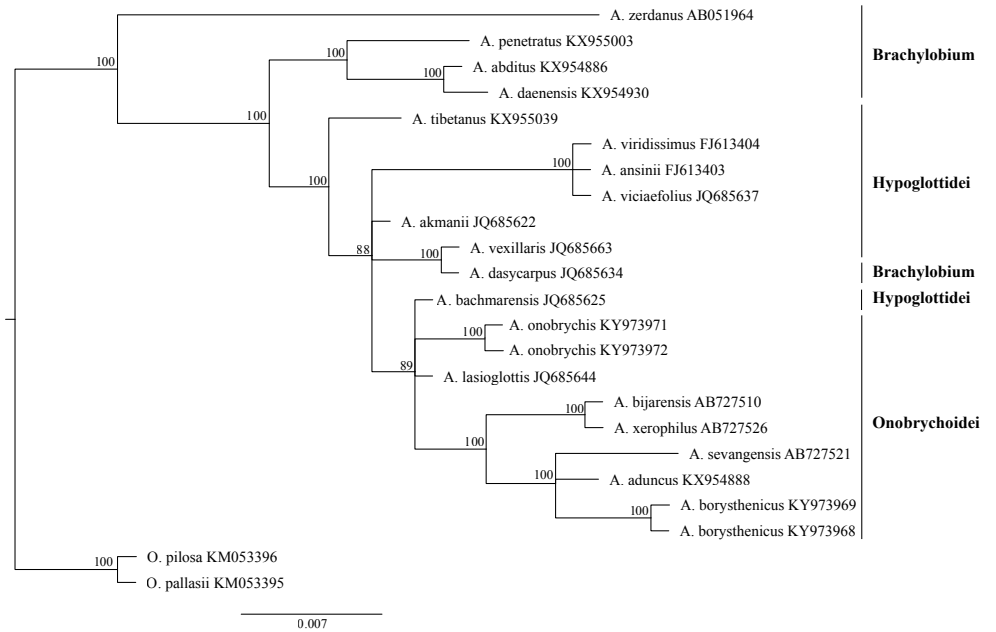

Figure 1. Bayesian phylogenetic tree of *Astragalus* ITS1-5.8S-ITS2 sequences. Numbers after the taxa names correspond to accession identifiers from GenBank. The number on each branch indicates the posterior probability (in percentages).

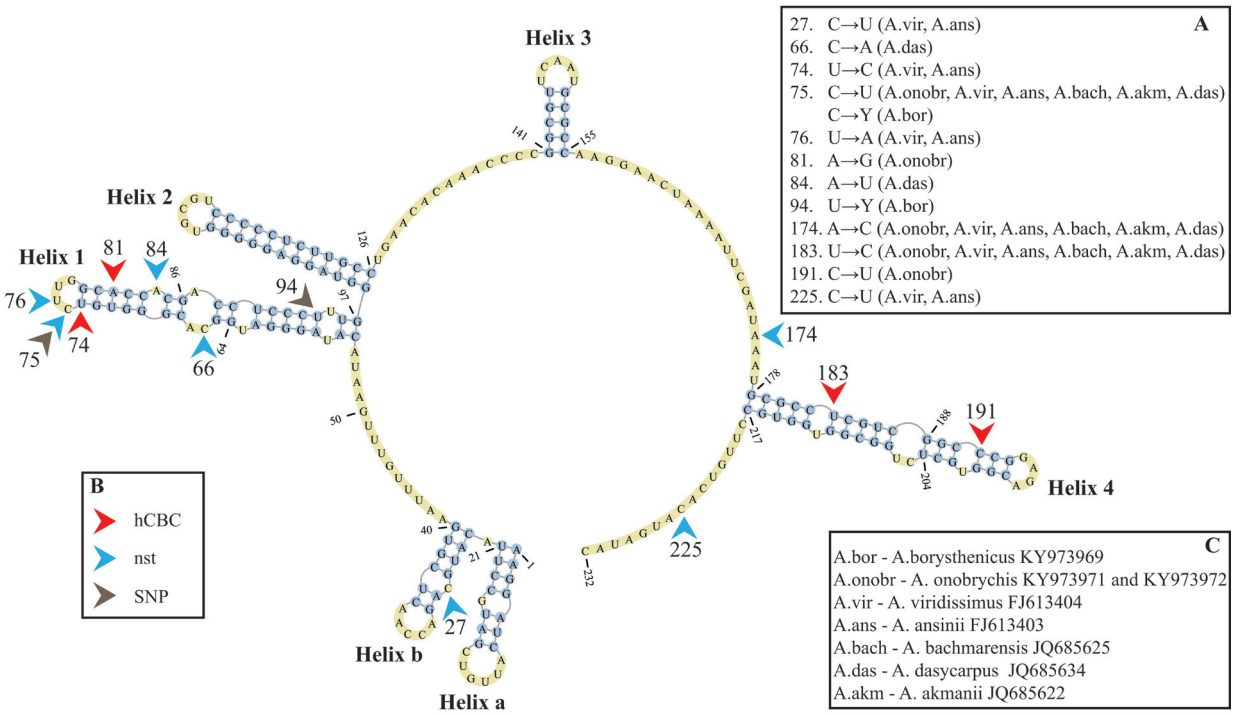

Figure 2. ITS1 secondary structure model of *A. borysthenicus* (KY973968) with differences from close taxa. Box A contains data on differences between related taxa and *A. borysthenicus* TBO, box B – type of changes, box C – abbreviations of *Astragalus* taxa.

of compensatory base changes. According to Coleman's concept (Coleman and Mai, 1997; Coleman, 2007), this fact indicates the possibility of sexual interaction on a gametic level between these taxa. However, differences in both ITS1 and ITS2 secondary structures are significant. hCBC and sst are considered taxonomically important changes (Coleman, 2000; Wolf et al., 2005; Moysiyenko et al., 2014). Thus, we used sst and hCBC as criteria for delineating variants of helices.

We obtained three variants of the first helix and three variants of the fourth helix of the ITS1 transcript. Two variants of the first helix, three variants of the third, and two variants of the fourth helix were revealed in ITS2 reconstructions (Figure 4).

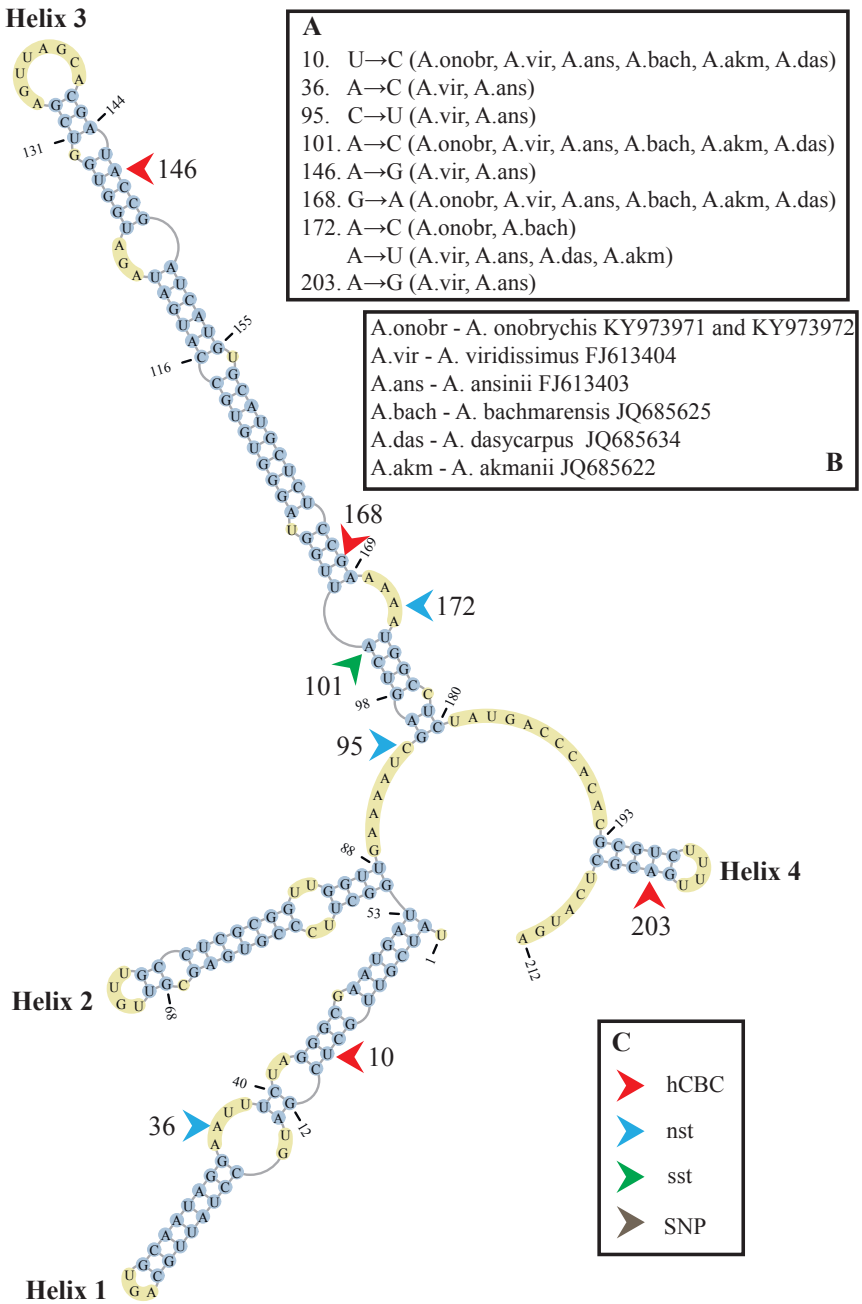

Figure 3. ITS2 secondary structure model of *A. borysthenicus* TBO (KY973968) with differences from close taxa. Box A contains data on differences between related taxa and *A. borysthenicus* TBO, box B – abbreviations of *Astragalus* taxa, box C – type of changes in secondary structure.

Table 4. Most informative variable sites in ITS1 and ITS2 secondary structures of the investigated taxa.

Taxa names	ITS1				ITS2				
	74	81	183	191	10	101	146	168	203
A. borysthenicus KY973968	U	A	U	C	U	A	A	G	A
A. borysthenicus KY973969	U	A	U	C	U	A	A	G	A
A. onobrychis KY973971	U	G	C	U	C	C	A	A	A
A. onobrychis KY973972	U	G	C	U	C	C	A	A	A
A. viridissimus FJ613404	C	A	C	C	C	C	G	A	G
A. ansinii FJ613403	C	A	C	C	C	C	G	A	G
A. akmanii JQ685622	U	A	C	C	C	C	A	A	A
A. bachmarensis JQ685625	U	A	C	C	C	C	A	A	A
A. dasycarpus JQ685634	U	A	C	C	C	C	A	A	A
Type of secondary change	hCBC	hCBC	hCBC	hCBC	hCBC	sst	hCBC	hCBC	hCBC

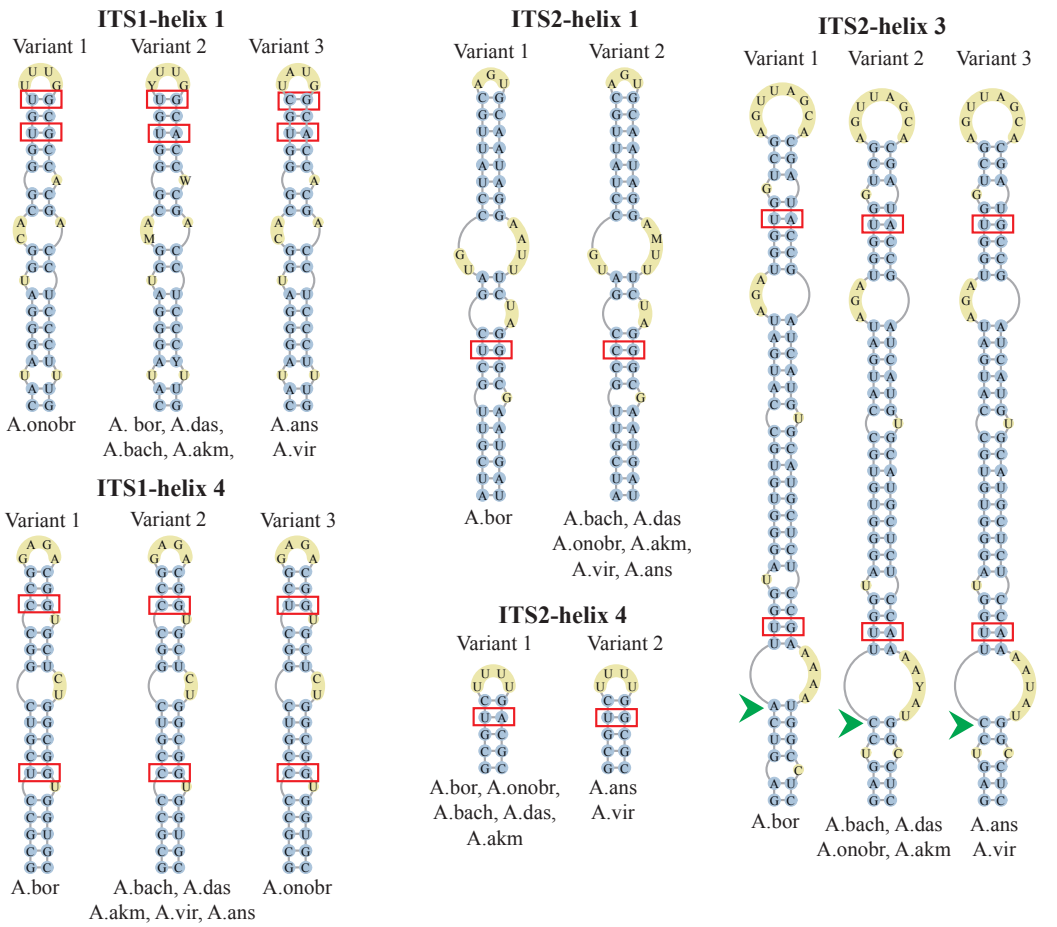

Figure 4. Helices variants of ITS1 and ITS2 that differentiate investigated taxa. The green arrow indicates sst, red boxes – hCBC. A list of abbreviations: A.bor – *A. borysthenicus* KY973968 and KY973969; A.onobr – *A. onobrychis* KY973971 and KY973972; A.bach – *A. bachmarensis* JQ685625; A.akm – *A. akmanii* JQ685622; A.das – *A. dasycarpus* JQ685634; A.vir – *A. viridissimus* FJ613404; A.ans – *A. ansinii* FJ613403.

Table 5. ITS1 and ITS2 secondary structures variants and corresponding OTU.

Taxa names	ITS1		ITS2			OTU
	Helix 1	Helix 4	Helix 1	Helix 3	Helix 4	
A. borysthenicus KY973968	Var. 2	Var. 1	Var. 1	Var. 1	Var. 1	OTU1
A. borysthenicus KY973969						
A. onobrychis KY973971, KY973972	Var. 1	Var. 3	Var. 2	Var. 2	Var. 1	OTU2
A. bachmarensis JQ685625	Var. 2	Var. 2	Var. 2	Var. 2		OTU3
A. akmanii JQ685622						
A. dasycarpus JQ685634						
A. viridissimus FJ613404	Var. 3			Var. 3	Var. 2	OTU4
A. ansinii FJ613403						

Thus, four independent operational taxonomic units (OTU) based on the results of the secondary structure analysis were identified: 1) *A. borysthenicus* sequences; 2) *A. onobrychis*; 3) *A. bachmarensis*, *A. akmanii*, and *A. dasycarpus*; 4) *A. ansinii* and *A. viridissimus* (Table 5).
OTU1 includes Ukrainian endemic *A. borysthenicus* that differs from other investigated taxa by three hCBCs (site 183 of ITS1; sites 10 and 168 of ITS2) and one unique sst (site 101 of ITS2). The *A. borysthenicus* specimen from Crimea (KY973968) has no ambiguous sites, while the specimen from Fedotova spit (KY973969) has two.

The specimen of *A. borysthenicus* KY973970 from Birjuchiy Island, previously declared as the neotype (Krytska et al., 1999), has five ambiguous sites. They are probably the result of nonconcerted evolution in nrDNA. However, considering the fact that one of the alternative alleles in ambiguous sites corresponds to "pure" *A. borysthenicus* from Crimea, and the other to *A. onobrychis* sequences, these sites may also be interpreted as SNPs. Therefore, the neotype specimen may demonstrate signs of ancient hybridization with *A. onobrychis* and probable introgression. Still, this specimen is not a true hybrid of *A. onobrychis* and *A. borysthenicus* because the two sites that differentiate these taxa (81 and 191 of the ITS1 sequence) do not have ambiguous nucleotides and match "pure" *A. borysthenicus*. This means that the specimen is not TBO; however, TBO is present among the other investigated samples of *A. borysthenicus* – KY973968.

Considering the specimen of *A. borysthenicus* from Crimea (KY973968) as a separate OTU and also TBO, it cannot be accepted as a synonym of *A. onobrychis*. It also clearly differs from Anatolian species *A. bachmarensis*, *A. akmanii*, *A. dasycarpus*, *A. viridissimus*, and *A. ansinii* in secondary structures of ITS transcripts and morphology. Moreover, *A. borysthenicus* is a separate clade according to phylogenetic reconstructions. Therefore, *A. borysthenicus* should be treated as a separate species.

OTU2 consists of two *A. onobrychis* sequences from Ukraine. Their sequences are identical and do not contain any ambiguous positions. Therefore, they represent "pure" *A. onobrychis*.

OTU3 consists of three samples – *A. bachmarensis*, *A. akmanii*, and *A. dasycarpus*. *A. bachmarensis* and *A. akmanii* differ in one nst in ITS2. *A. dasycarpus* differs from both of them by two nst in ITS1. These taxa have similar morphology. All of them are TBOs and are considered as endemic species (*A. bachmarensis* – West Caucasian; *A. dasycarpus* – Irano-Turanian, distributed only in eastern Turkey; *A. akmanii* –Irano-Turanian, also growing in southern Turkey) (Grossheim, 1930; Borisova, 1946; Güner et al., 2000; Uzun et al., 2009). These species are closely related, which is also confirmed by previously published phylogenetic trees (Dizkirici et al., 2013). It seems that they are a group of closely related species or intraspecific taxa.

OTU4 includes closely related *A. viridissimus* and *A. ansinii*, which differ by one indel and one nucleotide substitution in the 18S fragment and do not have any differences in ITS sequences.

Bayesian phylogenetic analysis, ITS1 and ITS2 secondary structure analysis, and some morphological data confirm the uniqueness of *A. borysthenicus* and allow considering it as a separate species. Thus, there is no reason to consider *A. borysthenicus* as a junior synonym of *A. onobrychis*.

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