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A systematic study of the *Thlaspi* s.l. taxa in sections *Nomisma, Thlaspi* and *Pterotropis* from Turkey based on fruit morphological and molecular data

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Running title: *A SYSTEMATIC STUDY OF* THLASPI *FROM TURKEY*

Abstract – The classification of *Thlaspi* s.l. is still problematic. Earlier phylogenetic research of the genus has focused on several small groups within *Thlaspi* s.str. and lacks detailed morphological observations. The relationships among Eurasian taxa and the value of fruit morphology in defining them have yet to be studied. The aim of this study was to analyze 22 taxa belonging to the *Nomisma*, *Thlaspi* and *Pterotropis* sections of *Thlaspi* s.l. from Turkey using maximum likelihood (ML) analysis of Internal Transcript Spacer (ITS) sequences. We also analyzed their fruit morphological features. According to the results, the examined taxa fell into 2 main clades. Moreover, clade II showed 3 sub-clusters. *Thlaspi huetii* and *T. aghricum* were the most distant taxa with a distance of 0.49%; however, *T. ochroleucum* and *T. violascens* were found to be 99% similar. According to ITS region data based on multiple populations of each taxon, *T. arvense*, *T. huetii*, *T. perfoliatum*, *T. violascens*, *T. cataonicum*, *T. elegans*, *T. rosulare* and *T. aghricum* were placed together in one cluster, which indicates that they are monophyletic. *Thlaspi elegans* was found to be a polyploid complex based on bootstrap (BS) (a resampling technique that uses replacement sampling to estimate statistics in a population) values, which varied widely among the studied *T. elegans* taxa (98, 65 and 49%). Fruit morphology also supported the inter-specific relationships based on molecular data, and relationships found by ITS region data were compatible with fruit type and geographic distribution. A diagnostic key based on fruit morphology is provided for the identification of the examined *Thlaspi* taxa.

Keywords: diagnostic key, fruit, ITS region, taxonomy, *Thlaspi*, Turkey

Introduction

The genus *Thlaspi* s.l. is a large and dynamic complex in the Brassicaceae family, that is widespread in Eurasia and North America and represented by more than 75 species (Karaismailoğlu and Erol 2018). In Turkey, it is represented by 36 taxa belonging to 6 sections, 22 of which are endemic. The first comprehensive study of the *Nomisma*, *Thlaspi* and *Pterotropis* sections was conducted by Hedge (1965). Sixteen taxa belonging to the sections specified in the study are included in the Flora of Turkey (Hedge 1965). Subsequent floristic studies have added *T. leblebicii* (Gemici and Görk 1995, Yıldırımlı 2001), *T. praecox* subsp. *praecox* Wulfen, *T. cariense* Carlström, *T. syriacum* Bornm., *T. aghricum* P.H. Davis & Kit Tan and *T. watsonii* P.H. Davis (Davis et al. 1988) to the Flora of Turkey, and today the specified sections are represented by 22 taxa.

Thlaspi consists of annual, biennial or perennial herbaceous plants with simple leaves and hairy or papillose stems. Sepals are bulging or non-saccate, the base of the sepals is often inclined and oblique with broad white membranaceous margins, while the oval petals are white, rose, lilac or yellowish. Filaments are narrow, straight or slightly curved. Nectar glands are present around the outer short stamens, but not on the long stamens in the middle. The ovary contains 2-16 ovules. The fruit is a dehiscent narrowly septate silicula or rarely a silique, strongly or weakly horizontally compressed, winged or not, with1-8 seeds per loculus. The septa is often wavy. Seeds may or may not contain mucilage. The embryonic rootlet is accumbent (resting on the edge of the cotyledons) (Hedge 1965).

The genus *Thlaspi* is a highly variable complex in Eurasia, which includes Turkey, and several researchers have conducted different types of studies on many of its taxa. First, some important characters improved the classification of this giant complex were found by Meyer (1973, 1991), who revised *Thlaspi* based on seed coat anatomy and embryology and transferred many of the taxa previously included in the genus to *Noccaea* Moench. Meyer (1973, 1991) also divided the genus *Thlaspi* s.l. into 12 genera (*Thlaspi* L., *Neurotropis* (DC.) F.K. Meyer, *Microthlaspi* F.K. Meyer, *Thlaspiceras* F.K. Meyer, *Noccidium* F.K. Meyer, *Kotschyella* F.K. Meyer, *Callothlaspi* F.K. Meyer, *Raparia* F.K. Meyer, *Noccaea* Moench, *Atropatenia* F.K. Meyer. *Vania* F.K. Meyer, *Masmenia* F.K. Meyer). According to this classification, 6 species remain in *Thlaspi* s. str. (*T. arvense* L., *T. huetii* Boiss., *T. watsonii* P.H. Davis, *T. kurdicum* Hedge, *T. alliaceum* and *T. ceratocarpon* Murray) (Meyer 1973 and 1991). The classification is not accepted by many scientists (Hedge 1965, Al-Shehbaz 1986, Karaismailoğlu 2018, Karaismailoğlu and Erol 2018, 2019, 2020) but has been accepted by others (Greuter and Burdet 1983). Although almost all of Meyer's classification was accepted by Greuter and Burdet (1983), this classification and the resulting new taxa were not accepted in the Flora of Turkey. Davis et al. (1988) rejected the genus fragmentation of Meyer (1973, 1991) and evaluated *Thlaspi* in the broad sense because they found the putative new taxa to be unanalyzable and, as a result, were unable to assess their proper placement. In addition, the Latin descriptions of many taxa were found to be insufficient by Davis et al. (1988). Some more recent studies using molecular markers (RubisCO, Chloroplast DNA, nuclear ribosomal DNA) clearly show that the boundaries of some genera (*Callothlaspi*, *Microthlaspi*, *Noccaea*, *Noccidium* and *Vania*) in Meyer's classification are unnatural (Mummenhoff and Koch 1994, Zunk et al. 1996, Mummenhoff et al. 1997, Koch et al. 1998, Koch and Mummenhoff 2001). According to Al-Shehbaz (2014), the systematic structure of Meyer (1973, 1991) is inherently incorrect and its inter-genus relations cannot be resolved. However, the study partially agreed with Meyer's classification at the genus level. According to Al-Shehbaz (2012), the genus *Noccaea*, a well-known taxonomic complex, contains most of the species transferred from *Thlaspi* s.l. Although he accepts that neither the genus boundaries nor the boundaries of the taxa within it can be fully resolved, he estimates the species diversity of *Noccaea* to be quite high with approximately 85 to 120 taxa (Al-Shehbaz 2012). Family-wide molecular phylogenetic studies place the genus *Noccidium* in the Camelineae tribe and indicate that the 10 genera (*Atropatenia*, *Callothlaspi*, *Kotschyella*, *Masmenia*, *Microthlaspi*, *Neurotropis*, *Raparia*, *Thlaspiceras*, *Vania*, *Noccaeopsis*). Meyer (1973, 1991) distinguished from *Thlaspi* are synonyms of *Noccaea* (Khosravi et al. 2009, Warwick et al. 2010). However, almost all of the taxa transferred are lacking in adequate field studies, with work most often based on herbarium specimens missing important organs. These studies also include few plants from Turkey, which is a center of diversity with many endemic *Thlaspi* taxa. It is therefore necessary to re-evaluate morphological characters in light of the intra-genus classification of Meyer (1973) and the transfers made in the following years (Meyer 1973, 1991), using molecular phylogenetic studies based on extensive field work.

Thlaspi is a difficult genus to study from a botanical point of view, mainly because it requires both individuals with flowers and ripe fruit for diagnosis. Many of the perennial species studied in the past, especially those with alpine distributions, were described from a few individuals, often without flowers or mature fruit. This makes the taxonomic status of many species uncertain (Hedge 1965, Karaismailoğlu 2018).

Fruits contain many typical morphological characteristics to distinguish taxa from each other, for instance shape, colour, size and microstructure (involving ultrastructure) often offer useful contributions to the taxonomy of angiosperms (Barthlott 1981, Karaismailoğlu 2017, 2019). Fruit morphology and surface micromorphology have been described as some of the best identification characters at the species and infra-generic levels in Brassicaceae (Hedge 1965, Davis et al. 1988, Karaismailoğlu 2018, 2019). It is difficult to morphologically distinguish *Thlaspi* from closely related genera without mature fruits. However, detailed studies of fruit morphology covering the genus as a whole have so far been lacking.

DNA sequencing has become one of the most important and widespread methods of investigating the phylogenetic status and taxonomic relationships among taxa in recent years. ITS (Internal Transcript Spacer) region is one of the most commonly used genomic regions in plant systematics, reasons for which include the presence of several types of Polymerase Chain Reaction (PCR) primer sets that can be used in different taxonomic groups (White et al. 1990, Gardes and Brus 1993, Gültepe 2014, Moorhouse-Gann et al. 2018), and the size of the region, less than 700 base pairs, which makes it easy to replicate and sequence (Gernandt et al. 2001). This region provides information useful in determining the phylogenetic relationships between taxa at the species and subspecies levels (Baldwin and Markos 1998, Gültepe 2014). Although a handful of studies have used different molecular markers to investigate several taxa, no comprehensive study of the ITS region has been conducted for *Thlaspi* s.l.

In this article, we present the most comprehensive sampling of *Nomisma*, *Thlaspi* and *Pterotropis* sections of *Thlaspi* s.l. from Turkey to date. Phylogenetic analyses based on nuclear ribosomal DNA (nrDNA) ITS sequence data using an extensive sample set are used to elucidate relationships between the taxa. We also support molecular data with fruit morphological character data in an attempt to better explain the fruit variation of *Thlaspi* s.l. species and to make a diagnostic key utilizing these taxonomically important characteristics.

Materials and methods

Sampling

In the current study, 22 species belonging to the *Nomisma*, *Thlaspi* and *Pterotropis* sections of the *Thlaspi* genus were collected as both flowering and ripe-fruited specimens from various phytogeographic regions in Turkey in March-July between 2013 and 2016. Specimens were numbered, pressed, arranged on herbarium sheets and deposited at Istanbul University Science Faculty Herbarium (ISTF) and Siirt University Flora and Fauna center (SUFAF) (On-line Suppl. Tab. 1.).

Macromorphological characteristics of the fruits such as shape, size, apical sinus, wing and septum structures were analyzed for 100 fruits, from 10 individuals of each species, using an Olympus SZX7 stereomicroscope and Kameram Imaging Software. For micromorphological examination of fruit surface ornamentation, we used a JEOL Neoscope-5000 Scanning Electron Microscope to examine samples fixed to stubs with silver epoxy and coated with platinum-gold mix (Karaismailoğlu and Erol 2018, Karaismailoğlu and Güner 2019).

DNA Extractions, ITS region amplification and sequencing process

Leaf samples were acquired from the field and ISTF and SUFAF herbaria, for a total of 33 accessions belonging to 22 species representing all three defined sections of *Thlaspi*. *Aethionema speciosum* Boiss. & A.Huet subsp. *compactum* Hartvig & Å.Strid [=*Ae. compactum* (Hartvig & Å.Strid) Yild.], which is closely related, was selected as the outgroup.

Total genomic DNA was isolated according to the Cetyl Trimethyl Ammonium Bromide (CTAB) method developed by Karaca et al. (2005). CTAB buffer is a mixture of Extraction Buffer (EB): (0.35 M sorbitol, 100 mM Tris-HCI (pH: 7.5), 5 mM EDTA (pH:7.5), %2 Tween, %1 Triton-X, %1 BME) and Lysis Buffer (LB): (200 mM Tris-HCI (pH:8.0), 50 mM EDTA (pH:8.0), 2M NaCI, %2 PPVP, %2 CTAB, %2 Triton-X, %2 BME). DNA quantitation was performed using a Thermo NanoDrop® Spectrophotometer. The ITS2 region sequences obtained from the genomic DNA was used as a template to amplify the ITS region with a MiniAmp Plus Thermal Cycler device using the primer pairs UniPlantF (5′-TGTGAATTGCARRATYCMG-3′) and UniplantR (5′-CCCGHYTGAYYTGRGGTCDC-3′) (Moorhouse-Gann et al. 2018). PCR was prepared in 25-µL volumes using the following reaction elements: 3 µL template DNA, 11.25 µL water, 2.5 µL 10X buffer, 1 µL each of primers (50 ng μL^{-1}), 4 µL $MgCl_2$ (2.5 mM), 1 µL dNTP mix (0.25 mM), 0.25 µL *Taq* DNA polymerase and 1 µL Bovine Serum Albumin (BSA).

PCR thermal cycle conditions were as follows: pre-denaturation = 95 °C (1 min.), DNA denaturation = 94 °C (1 min.), annealing = 55 °C, extension =72 °C (0.45 h), number of cycles =35 and final extension =72 °C (5 min.). Purification and sequencing outsourced to Genoks, (Istanbul, Turkey).

Bioinformatic analysis of sequences

We used ITS region base sequences of 33 accessions in our analyses. Sequences with poor quality reads throughout were sequenced again. Afterward, the first and last 30 bases were removed due to poor quality using the BioEdit program (Hall et al. 2011) and these sequences were not included in the main analysis. The obtained sequences were analyzed with the NCBI-BLAST algorithm to confirm they belong to the studied material. We then used Mega X version 10.0.05 (Kumar et al. 2018) to perform phylogenetic analyses. The sequences were first loaded and then aligned with the outgroup, *Ae. speciosum* subsp. *compactum*, included using the base sequence, and Clustal W (Larkin et al. 2007). After alignment, a phylogenetic tree was constructed to interpret the genetic similarities between taxa. All raw sequences were arranged in FASTA format for bioinformatics analysis. After editing the sequences, statistical analyses were performed in Mega X, using the predictive model algorithm to determine the most appropriate method for our study. Bootstrap values for 1000 replicates were obtained according to the maximum likelihood (ML) phylogenetic method.

Results

Structure of fruit in *Thlaspi*

All examined taxa have siliculae as fruit. Eight fruit shapes were observed: oval-circular, elliptical, oval, obcordate, cuneate-obcordate, cuneate, obcordate-triangular and rectangular (Fig. 1). The most common type is obcordate (in 10 taxa), while oval-circular (*T. arvense*), circular (*T. huetii*), cuneate-obcordate (*T. ochroleucum*), cuneate (*T. tatianae*), oval-obcordate (*T. cataonicum*) and rectangular (*T. elegans*) types are species specific (Fig. 1). Fruit sizes range from 4 mm (*T. perfoliatum*) to 18 mm (*T. arvense*) in length, from 2.5 mm (*T. tatianae*) to 16 mm (*T. arvense*) in width. *T. perfoliatum* has the smallest fruits and *T. arvense* has the largest. Fruit wing characteristics vary in width, wingtip structure and venation among taxa, except for *T. lilacinum*, which is not winged. The width of the wings varies between 0.3 mm (*T. tatianae*) and 5 mm (*T. arvense* and *T. orbiculatum*). Wing tips may be rotundate, obtuse or acute. The

most common form is rotundate (12 taxa), while acute is the least (4 taxa). The wing tips are the same length in all taxa studied except for *T. elegans*, which has different sized wing tips. The wings have reticulate surface venation, except for *T. orbiculatum*, which has parallel venation (Fig. 1, Tab. 1).

The apical sinus also differs in terms of structure and size in the taxa studied. It is absent in *T. lilacinum*. It may be broad or quite narrow, as in *T. watsonii*. Apical sinus length varies between 0.1 mm (*T. watsonii*) and 4 mm (*T. arvense*). The length of the fruit stylus and its relationship to apical sinus length are quite diverse among these taxa. Stylus length is between 0.1 mm (*T. arvense*, *T. huetii* and *T. kotschyanum*) and 4 mm (*T. watsonii*). Septum sizes vary from 4 mm (*T. huetii* and *T. perfoliatum*) to 11 mm (*T. arvense*, *T. orbiculatum* and *T. bulbosum*) in length, and from 0.5 mm (*T. microstylum*) to 4 mm (*T. lilacinum*) in width. The number of seeds per locus ranges from 2 (11 taxa) to 8 (*T. arvense*) (Fig. 1, Tab. 1).

Fruit surface ornamentation is categorized into 8 types: reticulate, rugose, areolate, favulariate, ruminate, straight, lineolate and ocellate. The most common types are rugose and ruminate (13 taxa). Areolate (*T. orbiculatum*), favulariate (*T. perfoliatum*), lineolate (*T. tatianae*) and ocellate (*T. aghricum*) ornamentation types are represented by one taxon each (On-line Suppl. Fig. 1, Tab. 1).

Phylogenetic relation of *Thlaspi* based on ITS region sequences

The ITS gene sequences of the 22 *Thlaspi* taxa studied were used in analyses, plus that of *Ae. speciosum* subsp. *compactum* as the outgroup. The bootstrap values of the phylogenetic tree obtained using the maximum likelihood (ML) phylogenetic method are shown in Fig. 2.

The dendrogram obtained from ITS data shows two different clusters, or clades I and II. Clade II has six sub-clusters: A, B, C1, C2, C3 and C4 (Fig. 2). Only *T. huetii* in the *Nomisma* section was placed in clade I. The other taxon of that section, *T. arvense*, was found in Cluster A of clade II, meaning that the *Nomisma* taxa preserve their existing systematic proximity. *T. orbiculatum* and *T. kotschyanum*, closely related in the *Thlaspi* section in the Flora of Turkey, remain closely linked to each other in Cluster B of clade II. Cluster C of clade II included 18 studied species and was further divided into four clusters: C1, C2, C3 and C4. Due to the aggregation of taxa in this cluster, it can be thought that other taxa originate from this cluster. *Thlaspi annuum* and *T. perfoliatum* from *Thlaspi* section are closely associated with each other in C1 cluster. Taxa from the *Thlaspi* and *Pterotropis* sections (*T. leblebicii*, *T. rosulare*, *T. lilacinum* and *T. watsonii*) are in C2. All of these are narrow endemics in Turkey. *T. ochroleucum*, *T. cariense*, *T. densiflorum*, *T. violascens*, *T. tatianae*, *T. cataonicum* and *T. syriacum* taxa are included in the C3 cluster. Common features among taxa in this cluster include generally obcordate-shaped siliculae and horizontally suppressed fruits. Cluster C4 consists of *T. bulbosum* and *T. microstylum*, belonging to *Thlaspi* section, plus *T. praecox* subsp. *praecox*, *T. elegans* and *T. aghricum* of the *Pterotropis* section. Although *T. bulbosum* and *T. praecox* subsp. *praecox* taxa are widely found in the European Flora, they have a very limited distribution in Turkey.

The dissimilarity matrix based on ITS region data is provided in on-line Suppl. Tab. 2. All examined taxa have differences ranging from 0.01 to 0.49%, except for the outgroup. *Thlaspi huetii* and *T. aghricum* are the most distinct taxa with a distance of 0.49% between them. *Thlaspi violascens* and *T. ochroleucum*-*T. violascens* had a 99% similarity to each other. The lengths of the nrDNA-ITS region ranged from 260 to 378 bp, and % GC contents varied between 45.91 and 54.73 (On-line Suppl. Tab. 3). Alignment of the ITS regions of all examined taxa resulted in a data set consisting of 324 base pair (bp). We found that 95 of this aligned data consisted of parsimony (informative) nucleotides.

Discussion

Fruit morphological characters provide valuable information regarding the evolutionary relationships of flowering plants (Corner 1976). In this study, fruit macro- and micromorphology generally varies across *Thlaspi* species. Meyer (1973, 1991, 2001, 2006) discusses fruit similarities within the genus and argues that the relationships among taxa are masked in fruit-based classification. However, we found ripe fruits do not show this similarity. Instead, mature fruit morphology ranged from broad to narrow, winged to wingless and rounded to acute to obtuse in the wing tips. In addition, the width and length of the apical sinus on the fruit, and the comparative length of the stylus are among characters that distinguish species. A thorough diagnostic key-based fruit characters is found at the end of the discussion section.

The examined *Thlaspi* taxa have overall very high similarity rates for the ITS region, which indicates that the genus has not fully completed the differentiation process. Previous studies conducted with various genera have shown that the ITS region contains a considerable number of parsimony informative nucleotides and therefore clearly reveals the relationship between taxa (Baldwin et al. 1998, Alvarez and Wendel 2003, Hughes et al. 2006). We targeted this region specifically because of its parsimony informative nucleotides and used the data to generate a phylogenetic tree (Fig. 2).

Many studies have shown that the ITS gene region offers remarkable solutions in explaining the relationships between taxa in some species-rich genera (Soltis and Soltis 1993, Soltis et al. 2008). The ITS region shows significant divergence between species but is often highly conserved within taxa, making it one of the most chosen genetic markers for species-level delimitation (Cheng et al. 2016). Moreover, the prospects of amplification from processed or aged plant materials are good due to the ITS region's large copy number (Balasubramani et al. 2010). ITS has been effectively used to differentiate taxa in diverse plant groups. According to our ITS data, the examined taxa are monophyletic and highly similar, which does not support previous taxa transfers to different genera, and all examined taxa should be evaluated under the same genus. A similar result was reported in a dendrogram created by evaluating 215 macromorphological, micromorphological and anatomical characters (Karaismailoğlu 2018), as well as a cladogram based on the detailed examination of palynological characters of these taxa (Karaismailoğlu and Erol 2019).

Taxa belonging to the *Nomisma* section are easily distinguished from others by their broad wings and oval, elliptical or circular fruits. This data supports distinguishing this section from others according to fruit shape, as done by Schulz (1936) and Hedge (1965) in Flora of Turkey, who established the section using fruit shape and supported it with other classifications. *Thlaspi orbiculatum* and *T. kotschyanum* taxa are morphologically very similar in flower structure and broadly obcordate-shaped fruit; however, a contrast between the parallel-veined fruit wings of *T. orbiculatum* and the reticulate venation of *T. kotschyanum* distinguishes the two. Plant height, leaf size and seed characteristics used in Karaismailoğlu (2018) also clearly differentiate these taxa. Clusters in this group are consistent with the key characters and descriptions in Flora of Turkey described by Hedge (1965) and Davis et al. (1988) (Fig. 2). *Thlaspi annuum* and *T. perfoliatum* are morphologically similar in that their petals are in two segments, inner and outer. In the subset formed by *T. perfoliatum* taxa, the length of the petals in the outer segment is equal to those of the inner segment, while in *T. annuum* the petals in the outer segment are longer than in the inner segment (Karaismailoğlu 2018). Surprisingly, *T. leblebicii* and *T. watsonii* branch from the same place in the dendrogram because these taxa are quite different in appearance and distributed in different phytogeographic regions. The dendrogram positions of *T. rosulare, T. lilacinum* and *T. watsonii* are compatible with their positions in the Flora of Turkey. *Thlaspi ochroleucum, T. densiflorum* and *T. violascens*, which are morphologically similar, are closely related but separated from each other by their BS values. *Thlaspi bulbosum* and *T. praecox* subsp. *praecox* are similar to each other in that they have underground stem metamorphoses namely rhizomes and tubers, obcordate-shaped silicula, narrow or wide fruit

wings, rounded wing tips and fruit styluses that generally exceed the apical sinus, all of which are also used as key characters in the Flora of Turkey (Hedge 1965, Davis et al. 1988). On the other hand, *T. bulbosum* differs from *T. praecox* subsp. *praecox* in GC% (51.38 in *T. bulbosum*, 50.99 in *T. praecox* subsp. *praecox*) and ITS region length (323 bp in *T. bulbosum*, 300 bp in *T. praecox* subsp. *praecox*).

The rankings and relationships of taxa in the dendrogram obtained by molecular phylogenetic data are also supported by taxa descriptions in the Flora of Turkey (Fig. 2). On the other hand, we see no parallel between the sections created using morphological data and molecular data. Based on ITS comparisons, the close proximity between the *Nomisma* section taxa (*T. arvense* and *T. huetii*) is preserved. On the other hand, we see a gradual transition in taxa belonging to the *Thlaspi* and *Pterotropis* sections, similar to what has been observed in previous detailed studies on the genus (Karaismailoğlu 2018, Karaismailoğlu and Erol 2018, 2019, 2020, Karaismailoğlu and Fidan 2022). This shows that distinguishing sections based on fruit morphology is artificial and not taxonomically beneficial.

According to ITS data, *T. elegans* is a non-monophyletic polyploid complex. The BS values among the studied *T. elegans* taxa differ considerably (98%, 65%, 49%,). Also, ITS data from more than one population of *T. arvense*, *T. huetii*, *T. perfoliatum*, *T. violascens*, *T. cataonicum*, *T. elegans*, *T. rosulare* and *T. aghricum* indicates that they are same arm taxa, and thus monophyletic.

Thlaspi is a systematically problematic genus because of the presence of many morphologically similar species. *Thlaspi arvense-T. huetii*, *T. orbiculatum-T. kotschyanum*, *T. violascens-T. densiflorum* and *T. lilacinum-T. watsonii* are taxon pairs that are very similar to each other in terms of macromorphology. However, they are easily separated using the BS values of our ITS-based phylogenetic tree, telling us that despite the high morphological similarity between species, they can be differentiated by molecular methods.

This study, including a phylogenetic comparison of the ITS region, places the studied taxa into a natural systematic group due to high base sequence similarity. As in previous detailed morphological, anatomical, palynological and cytological studies (Karaismailoğlu 2018, Karaismailoğlu and Erol 2018, 2019, 2020, Karaismailoğlu and Fidan 2021) on the same taxa, we found that these taxa should be treated as part of *Thlaspi* s.l.

A diagnostic key for taxa studied based on fruit morphological characteristics

1. Fruit shapes are oval, circular, oval-circular, elliptical, cuneate or rectangular…..……. 2
2. Oval, oval-circular, circular or elliptical…….…... 3
3. Oval, oval-circular…….… 4
4. Oval-circular…….....…… *T. arvense*
4. Oval……..... 5
5. Stylus exceeds apical sinus…….....……...….. *T. watsonii*
5. Stylus does not exceed apical sinus……...……...……. *T. rosulare*
3. Elliptical……...……..… 6
6. Apical sinus absent……..…….....……..… *T. lilacinum*
6. Apical sinus present……...……...…….……. *T. huetii*
2. Cuneate or rectangular…….…….. 7
7. Cuneate…..…… *T. tatianae*
7. Rectangular……...……..……. *T. elegans*
1. Fruit shapes are obcordate, cuneate-obcordate, oval-obcordate or obcordate triangular….. 8
8. Cuneate-obcordate, oval-obcordate or obcordate……...……...…… 9
9. Cuneate-obcordate or oval-obcordate…….. 10
10. Cuneate-obcordate……...…….…..…….. *T. ochroleucum*

10. Oval-obcordate……..……... *T. cataonicum*
9. Obcordate…….…… 11
11. Fruit wings with parallel veins…….…….. *T. orbiculatum*
11. Fruit wings with reticulate veins…….……...… 12
12. Stylus exceeds apical sinus…….....…… 13
13. Wing tips are obtuse; septum width is 1.5-2 mm…….…….. *T. aghricum*
13. Wing tips are rotundate; septum width is 3 mm……...… *T. cariense*
12 .Stylus does not exceed apical sinus or is the same length……..……..…. 14
14. Stylus length >1 mm…….……. 15
15. Septum width is 0.5-1.5 mm; ornamentation type is reticulate……. *T. microstylum*
15. Septum width is 2-3 mm; ornamentation type is rugose…… *T. bulbosum*
14. Stylus length ≤1 mm…….…… 16
16. The number of seeds at each locus is 2-6…….……. 17
17. 6 seeds at each locus …..……..…….. ……..... *T. kotschyanum*
17. 2-5 seeds at each locus…….,,,… 18
18. Ornamentation type is favulariate or straight…….…… 19
19. Favulariate……..……..…... *T. perfoliatum*
19. Straight…….…… *T. praecox* subsp. *praecox*
18. Ornamentation type is rugose or ruminate…….…… 20
20. Rugose…….....…..… *T. annuum*
20. Ruminate…….…….... *T. leblebicii*
8. Obcordate-triangular…….…… 21
21. Wing tips are rotundate or obtuse……...…… 22
22. Ornamentation type is rugose……..……..…… *T. syriacum*
22. Ornamentation type is ruminate……...……..… *T. violascens*
21. Wing tips are acute……..……...…… *T. densiflorum*

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Tab. 1. Fruit macro- and micromorphological features of the *Thlaspi* taxa (L: length, W: width, mm: millimeter). The measurements were made on 100 fruits for each taxon.

Taxa	Fruit	Fruit sizes		Wing			Apical sinus		Stylus	Septum sizes		Number of seeds	Surface
	shape	L (mm)	W (mm)	W (mm)	Tip	Veining	Structure	L (mm)	length (mm)	L (mm)	W (mm)	in each locus	ornamentation
T. arvense	Oval-Circular	10–18	10–16	2–5	Rotundate	Reticulate	Narrow	2–4	0.1–0.2	7–11	1–2	4–8	Reticulate
T. huetii	Circular	6–10	5–10	1–2	Rotundate	Reticulate	Narrow	1.5–2	0.1–0.2	4–7	1.5–2	2–4	Rugose
T. orbiculatum	Obcordate	10–12	13–15	2–5	Rotundate	Parallel	Narrow	1–3	0.4–0.5	8–11	1–1.5	4–6	Areolate
T. kotschyanum	Obcordate	7–14	8–15	2–4	Rotundate	Reticulate	Narrow	2–3	0.1	6–10	2–3	6	Reticulate
T. perfoliatum	Obcordate	4–6	4–7	0.5–1.2	Rotundate	Reticulate	Broad	1–3	0.2–0.4	4–6	1–3	3–5	Favulariate
T. microstylum	Obcordate	6–10	3–7	1–2.5	Obtuse	Reticulate	Narrow	1–1.5	1.1–1.5	6–8	0.5–1.5	2–4	Slightly reticulate
T. annuum	Obcordate	5–8	3–6	1–2	Rotundate	Reticulate	Broad	0.8–1.1	0.8–1	4.5–7	1–2	3–5	Rugose
T. bulbosum	Obcordate	8–11	6–10	1–3	Rotundate	Reticulate	Broad	2–3	2–3	8–11	2–3	2	Rugose
T. leblebicii	Obcordate	6–12	4–7	1–2	Rotundate	Reticulate	Narrow	1–2	0.2–0.5	6–8	1.5–2.5	2	Ruminate
T. ochroleucum	Cuneate-obcordate	5–8	4–6	0.5–1	Acute	Reticulate	Broad	0.5–1	2–3	5–6	1–2	4	Straight
T. praecox subsp. *praecox*	Obcordate	5–8	3–4	1–2	Rotundate	Reticulate	Broad	0.5–1	0.5–1	5–7	1–2	2–4	Straight
T. cariense	Obcordate	9–12	4–6	1–2	Rotundate	Reticulate	Broad	0.4–0.8	1–3	6–8	3	2–4	Rugose
T. violascens	Obcordate-triangular	8–10	4–6	1–2	Rotundate	Reticulate	Narrow	1.5–2	1–2	6–7	1.5–2	4–5	Ruminate
T. densiflorum	Obcordate-triangular	8–9	3.5–4	1–2	Acute	Reticulate	Narrow	1.5–2.2	1.5–2	7–8	1.5–2	4	Ruminate
T. tatianae	Cuneate	6–10	2.5–4	0.3–1	Obtuse	Reticulate	Broad	1–1.8	0.3–0.5	6–7	0.8–1.2	5–6	Lineolate
T. cataonicum	Oval-obcordate	10–12	3–4	0.5–1	Acute	Reticulate	Broad	2–2.5	2–2.3	7–8	1.5–2.1	5–6	Rugose
T. syriacum	Obcordate-triangular	6–8	3–4	0.5–1	Obtuse	Reticulate	Broad	0.5–0.7	1.5–2	5–6	1–1.5	2–4	Rugose
T. elegans	Rectangular	5–7	3–4.5	0.5–1.5	Acute	Reticulate	Broad	0.8–1.1	1–1.5	5–6	1–2	2–4	Ruminate
T. rosulare	Oval	7–9	5–6	0.7–1.2	Obtuse	Reticulate	Narrow	1–1.2	0.2–0.5	5–6	2.5–3	2	Ruminate
T. lilacinum	Elliptical	5–9	3–4.5	–	–	–	–	–	2–2.5	5–8	3–4	4–6	Rugose
T. aghricum	Obcordate	8–12	5–9	1–2.2	Obtuse	Reticulate	Broad	0.5–0.7	1.5–2	5–6	1–1.5	2–4	Ocellate
T. watsonii	Oval	5–7	3–4	1–2	Rotundate	Reticulate	Absent or Narrow	0.1–0.2	3.5–4	5–6	2–2.5	2–6	Ruminate

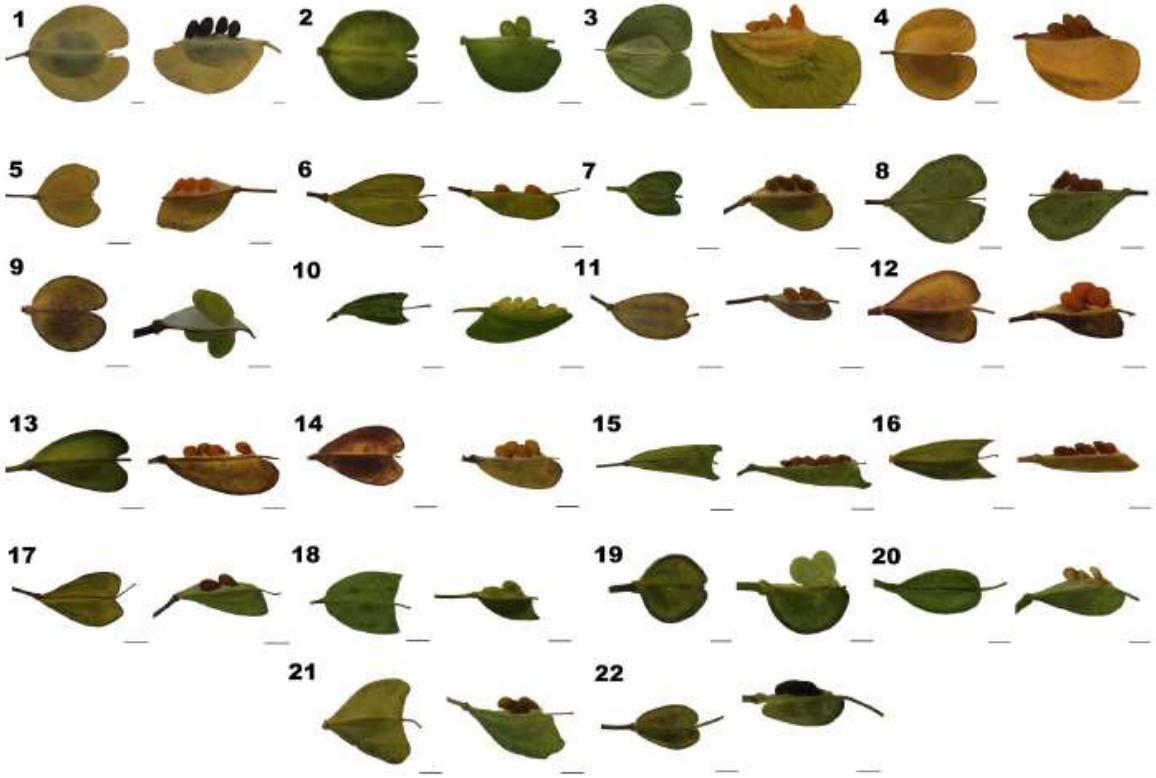


Fig. 1. Fruits of the *Thlaspi* taxa (two images per taxon; 1 – fruit general appearance, 2 – number of seeds per locus): 1 – *T. arvense*, 2 – *T. huetii*, 3 – *T. orbiculatum*, 4 – *T. kotschyanum*, 5 – *T. perfoliatum*, 6 – *T. microstylum*, 7 – *T. annuum*, 8 – *T. bulbosum*, 9 – *T. leblebicii*, 10 – *T. ochroleucum*, 11 – *T. praecox* subsp. *praecox*, 12 – *T. cariense*, 13 – *T. violascens*, 14 – *T. densiflorum*, 15 – *T. tatianae*, 16 – *T. cataonicum*, 17 – *T. syriacum*, 18 – *T. elegans*, 19 – *T. rosulare*, 20 – *T. lilacinum*, 21 – *T. aghricum*, 22 – *T. watsonii*. Scale bars: 2 mm.

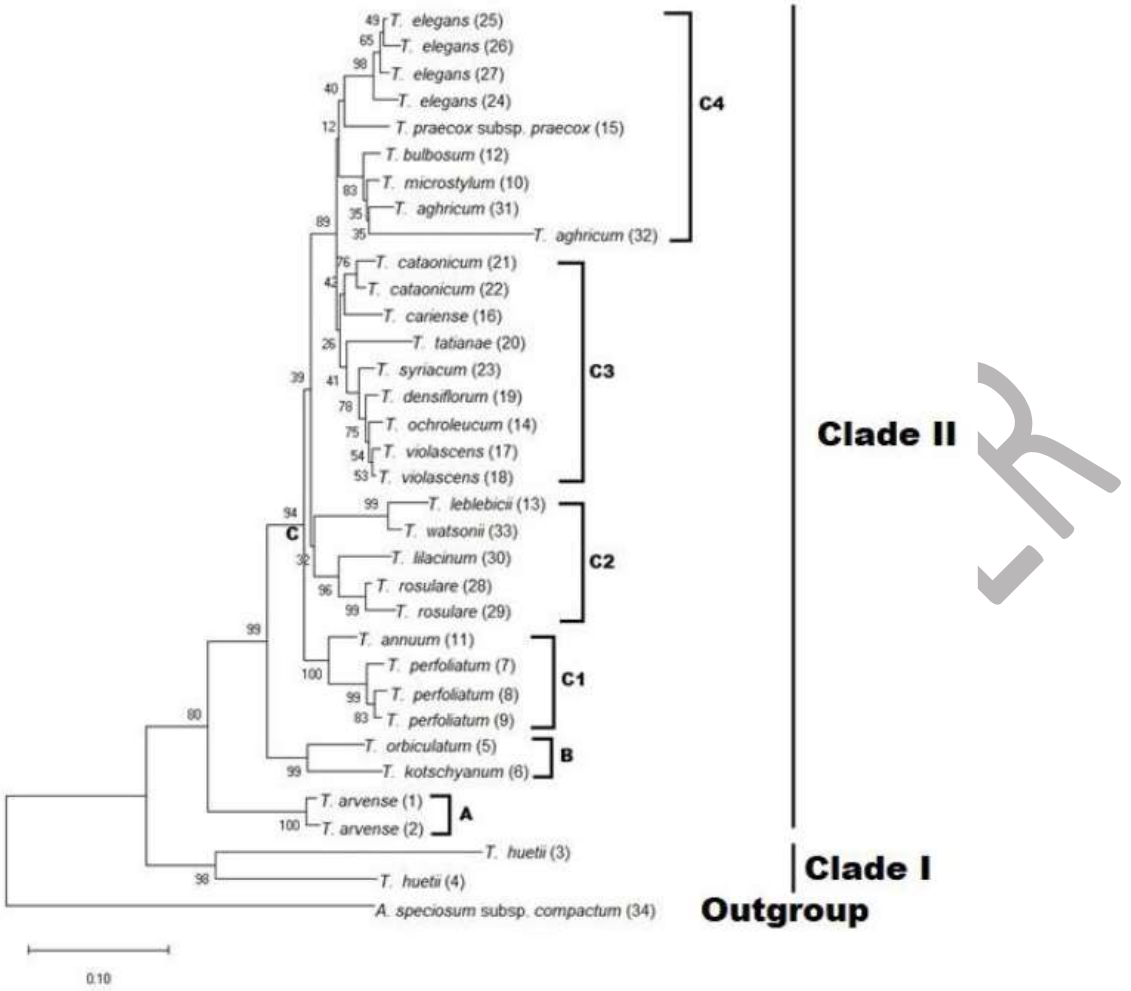


Fig. 2. Phylogenetic tree for representatives of the examined *Thlaspi* taxa based on ITS region data. Numbers at nodes show the bootstrap values. Numbers in parentheses indicate accession numbers. See On-line Suppl. Tab. 1 for locality data.

On-line Suppl. Tab. 1. The *Thlaspi* taxa used in the study and their locations (*=endemic taxon).

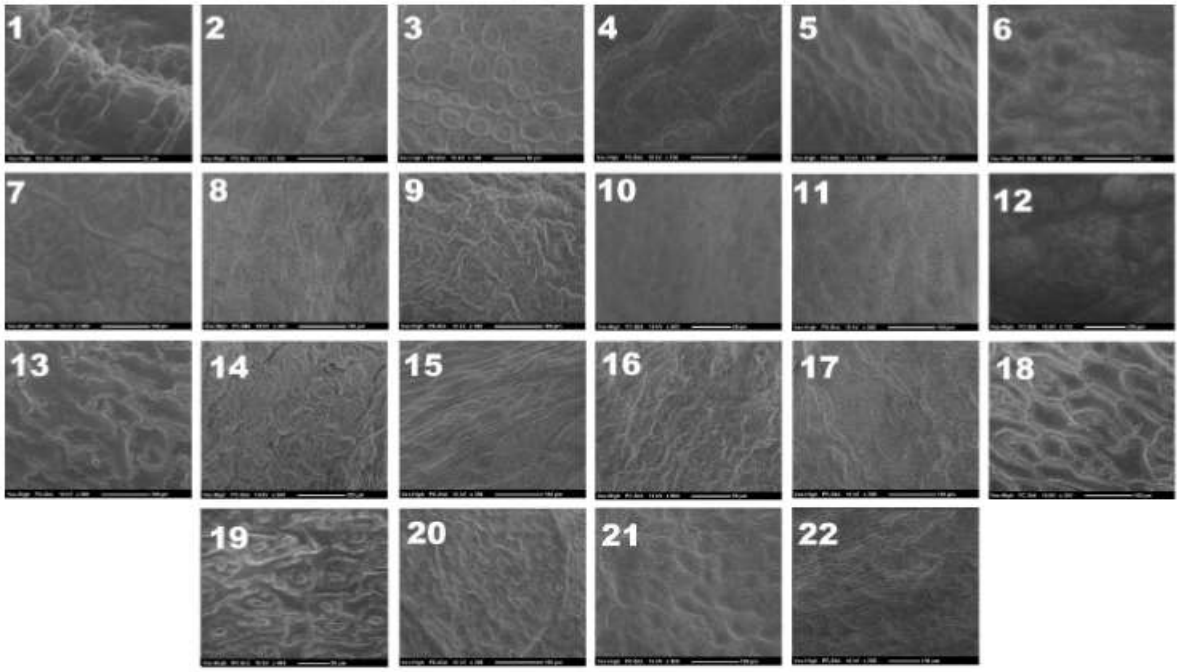
No	Section	Taxa	Location	Collection number
1	*Nomisma*	*Thlaspi arvense* L.	Samsun, Kavak	Karaismailoğlu 139
2		*T. arvense*	Rize, İkizdere	Karaismailoğlu 172
3		*T. huetii* Boiss.	Ağrı, Merkez	Karaismailoğlu 168
4		*T. huetii*	Artvin, Şavşat	Karaismailoğlu 66
5	*Thlaspi*	*T. orbiculatum* Stev.	Artvin, Ardanuç	Karaismailoğlu 254
6		*T. kotschyanum* Boiss. & Hohen.	Maraş, Göksun	Karaismailoğlu 202
7		*T. perfoliatum* L.	Gümüşhane, Kürtün	Karaismailoğlu 103
8		*T. perfoliatum*	Tekirdağ, Merkez	Karaismailoğlu 109
9		*T. perfoliatum*	Bolu, Abant	Karaismailoğlu 132a
10		**T. microstylum* Boiss.	Osmaniye, Düziçi	Karaismailoğlu 129
11		*T. annuum* Koch	Amasya, Taşova	Karaismailoğlu 360
12		*T. bulbosum* Spruner ex Boiss.	Kahramanmaraş	Karaismailoğlu 208
13		**T. leblebicii* Gemici & Görk	Muğla, Köyceğiz	Karaismailoğlu 192
14	*Pterotropis*	*T. ochroleucum* Boiss.	Hatay, Dörtyol	Karaismailoğlu 240
15		*T. praecox* Wulfen subsp. *praecox*	Kırklareli, Dereköy	Karaismailoğlu 219
16		**T. cariense* A.Carlström	Muğla, Köyceğiz	Karaismailoğlu 193
17		**T. violascens* Boiss.	Osmaniye, Düziçi	Karaismailoğlu 129
18		**T. violascens*	Osmaniye, Düziçi	Karaismailoğlu 225
19		**T. densiflorum* Boiss. & Kotschy	Hatay, Dörtyol	Karaismailoğlu 241
20		*T. tatianae* Bordz.	Van, Güzeldere	Karaismailoğlu 187
21		*T. cataonicum* Reuter	Adana, Saimbeyli	Karaismailoğlu 124
22		*T. cataonicum*	Kahramanmaraş, Göksun	Karaismailoğlu 233
23		**T. syriacum* Bornm.	Osmaniye, Hasanbeyli	Karaismailoğlu 222
24		**T. elegans* Boiss.	Osmaniye, Zorkun	Karaismailoğlu 178
25		**T. elegans*	Osmaniye, Zorkun	Karaismailoğlu 223a
26		**T. elegans*	Osmaniye, Düziçi	Karaismailoğlu 226
27		**T. elegans*	Kahramanmaraş, Göksun	Karaismailoğlu 234
28		**T. rosulare* Boiss. & Bal.	Niğde, Çamardı	Karaismailoğlu 173
29		**T. rosulare*	Niğde, Çamardı	Karaismailoğlu 268
30		**T. lilacinum* Boiss. & Huet.	Rize, İkizdere	Karaismailoğlu 217
31		**T. aghricum* P.H.Davis & Kit Tan	Ağrı, Hamur	Karaismailoğlu 162
32		**T. aghricum*	Ağrı, Hamur	Karaismailoğlu 198
33		**T. watsonii* P.H.Davis	Van, Güzeldere	Karaismailoğlu 264
34	Outgroup	*Aethionema speciosum* subsp. *compactum* Hartvig & Strid	Muğla, Köyceğiz	Karaismailoğlu 260

On-line Suppl. Tab. 2. Dissimilarity matrix of the *Thlaspi* taxa according to ITS region (See On-line Suppl. Tab. 1 for taxa numbers and locations).

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
1																																		
2	0.02																																	
3	0.30	0.30																																
4	0.24	0.24	0.31																															
5	0.19	0.16	0.40	0.33																														
6	0.22	0.20	0.42	0.34	0.10																													
7	0.21	0.20	0.41	0.35	0.14	0.16																												
8	0.21	0.21	0.39	0.33	0.16	0.17	0.03																											
9	0.20	0.19	0.40	0.35	0.13	0.16	0.02	0.02																										
10	0.21	0.22	0.41	0.34	0.16	0.17	0.11	0.11	0.12																									
11	0.18	0.19	0.39	0.33	0.13	0.15	0.05	0.07	0.06	0.09																								
12	0.22	0.21	0.42	0.34	0.16	0.16	0.11	0.12	0.11	0.02	0.09																							
13	0.21	0.21	0.40	0.35	0.19	0.19	0.16	0.15	0.16	0.13	0.13	0.14																						
14	0.20	0.20	0.42	0.33	0.16	0.16	0.12	0.11	0.11	0.07	0.09	0.07	0.13																					
15	0.22	0.23	0.42	0.36	0.16	0.18	0.13	0.11	0.13	0.07	0.11	0.07	0.14	0.06																				
16	0.21	0.21	0.42	0.35	0.15	0.16	0.12	0.12	0.11	0.06	0.11	0.06	0.14	0.07	0.06																			
17	0.21	0.20	0.42	0.34	0.16	0.16	0.12	0.11	0.11	0.07	0.10	0.07	0.13	0.01	0.06	0.07																		
18	0.21	0.20	0.43	0.35	0.15	0.16	0.11	0.11	0.11	0.06	0.09	0.07	0.13	0.02	0.05	0.08	0.01																	
19	0.21	0.20	0.41	0.33	0.15	0.16	0.12	0.12	0.11	0.06	0.09	0.07	0.14	0.02	0.05	0.07	0.02	0.02																
20	0.22	0.21	0.41	0.34	0.16	0.17	0.14	0.13	0.13	0.09	0.12	0.09	0.17	0.08	0.09	0.09	0.06	0.07	0.07															
21	0.20	0.21	0.42	0.33	0.16	0.16	0.11	0.10	0.11	0.06	0.09	0.05	0.13	0.05	0.07	0.05	0.05	0.05	0.05	0.08														
22	0.19	0.21	0.41	0.33	0.15	0.16	0.10	0.10	0.10	0.05	0.08	0.05	0.13	0.04	0.07	0.04	0.04	0.04	0.04	0.07	0.02													
23	0.21	0.21	0.42	0.34	0.16	0.17	0.11	0.10	0.11	0.07	0.09	0.07	0.13	0.03	0.06	0.05	0.03	0.03	0.03	0.07	0.04	0.04												
24	0.22	0.23	0.43	0.35	0.16	0.18	0.13	0.11	0.13	0.08	0.11	0.07	0.15	0.07	0.07	0.08	0.07	0.08	0.07	0.10	0.07	0.07	0.07											
25	0.20	0.21	0.42	0.33	0.15	0.18	0.12	0.12	0.11	0.07	0.10	0.06	0.15	0.07	0.07	0.07	0.07	0.07	0.06	0.09	0.07	0.06	0.07	0.03										
26	0.21	0.21	0.41	0.35	0.15	0.18	0.12	0.12	0.10	0.06	0.09	0.07	0.15	0.09	0.07	0.09	0.08	0.09	0.08	0.12	0.07	0.06	0.06	0.03	0.02									
27	0.20	0.22	0.42	0.34	0.15	0.18	0.12	0.12	0.11	0.07	0.09	0.06	0.15	0.07	0.07	0.07	0.07	0.07	0.06	0.10	0.08	0.06	0.07	0.04	0.01	0.02								
28	0.18	0.19	0.41	0.33	0.12	0.15	0.11	0.11	0.11	0.11	0.10	0.09	0.12	0.09	0.11	0.10	0.09	0.10	0.09	0.12	0.10	0.08	0.10	0.11	0.02	0.10	0.09							
29	0.20	0.20	0.40	0.33	0.15	0.17	0.12	0.11	0.12	0.11	0.10	0.12	0.14	0.13	0.11	0.12	0.11	0.13	0.11	0.13	0.11	0.10	0.11	0.11	0.11	0.14	0.12	0.03						
30	0.20	0.22	0.43	0.33	0.15	0.15	0.15	0.14	0.14	0.11	0.11	0.11	0.15	0.11	0.12	0.11	0.11	0.11	0.11	0.13	0.11	0.09	0.11	0.13	0.11	0.12	0.11	0.07	0.08					
31	0.21	0.22	0.42	0.33	0.17	0.18	0.13	0.13	0.12	0.03	0.10	0.04	0.15	0.08	0.07	0.09	0.07	0.08	0.07	0.10	0.07	0.06	0.07	0.08	0.07	0.08	0.07	0.10	0.13	0.11				
32	0.29	0.29	0.49	0.33	0.28	0.27	0.20	0.20	0.19	0.13	0.18	0.14	0.25	0.17	0.19	0.19	0.16	0.19	0.17	0.19	0.18	0.16	0.17	0.18	0.17	0.20	0.17	0.19	0.22	0.19	0.14			
33	0.21	0.21	0.40	0.35	0.17	0.18	0.14	0.14	0.14	0.11	0.12	0.11	0.04	0.11	0.12	0.11	0.12	0.12	0.12	0.14	0.11	0.11	0.12	0.13	0.13	0.13	0.13	0.10	0.12	0.12	0.12	0.21		
34	0.65	0.66	0.70	0.65	0.64	0.64	0.65	0.64	0.65	0.64	0.65	0.65	0.66	0.65	0.64	0.64	0.65	0.64	0.65	0.63	0.63	0.63	0.63	0.65	0.65	0.64	0.65	0.67	0.67	0.66	0.64	0.65	0.66	

On-line Suppl. Tab. 3. ITS lengths (bp) and GC% of the *Thlaspi* taxa (bp: base pair, See On-line Suppl. Tab. 1 for taxa numbers and locations).

Taxa	GC%	bp
T. arvense (1)	46.05	330
T. arvense (2)	50.43	378
T. huetii (3)	45.91	331
T. huetii (4)	46.33	328
T. orbiculatum (5)	49.24	331
T. kotschyanum (6)	50.14	331
T. perfoliatum (7)	55.58	331
T. perfoliatum (8)	54.73	327
T. perfoliatum (9)	54.37	331
T. microstylum (10)	51.25	318
T. annuum (11)	52.77	324
T. bulbosum (12)	51.38	323
T. leblebicii (13)	50.30	324
T. ochroleucum (14)	50.75	327
T. praecox subsp. *praecox* (15)	50.99	300
T. cariense (16)	52.25	331
T. violascens (17)	51.53	324
T. violascens (18)	50.45	327
T. densiflorum (19)	51.38	323
T. tatianae (20)	52.30	325
T. cataonicum (21)	51.25	318
T. cataonicum (22)	51.72	319
T. syriacum (23)	51.40	319
T. elegans (24)	50.79	313
T. elegans (25)	49.83	323
T. elegans (26)	49.99	334
T. elegans (27)	50.61	322
T. rosulare (28)	52.00	323
T. rosulare (29)	53.13	335
T. lilacinum (30)	51.07	323
T. aghricum (31)	48.92	327
T. aghricum (32)	47.30	260
T. watsonii (33)	50.77	321
Aethionema speciosum subsp. *compactum* (34)	47.07	342



On-line Suppl. Fig. 1. The surface micromorphological structures of fruits of the *Thlaspi* taxa: 1 – *T. arvense*, 2 – *T. huetii*, 3 – *T. orbiculatum*, 4 – *T. kotschyanum*, 5 – *T. perfoliatum*, 6 – *T. microstylum*, 7 – *T. annuum*, 8 – *T. bulbosum*, 9 – *T. leblebicii*, 10 – *T. ochroleucum*, 11 – *T. praecox* subsp. *praecox*, 12 – *T. cariense*, 13 – *T. violascens*, 14 – *T. densiflorum*, 15 – *T. tatianae*, 16 – *T. cataonicum*, 17 – *T. syriacum*, 18 – *T. elegans*, 19 – *T. rosulare*, 20 – *T. lilacinum*, 21 – *T. aghricum*, 22 – *T. watsonii*.