

An attempt to solve the taxonomic confusion of *Thlaspiceras* F.K.Mey. complex (*Noccaea* Moench-Brassicaceae) with ISSRs

Kurtuluş ÖZĞİŞİ¹, Atila OCAK¹, Burcu TARIKAHYA-HACIOĞLU^{2*}

¹Department of Biology, Faculty of Science and Letters, Eskişehir Osmangazi University Eskişehir, Turkey

²Department of Biology, Faculty of Science, Hacettepe University, Ankara, Turkey

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Abstract: Meyer separated *Thlaspi* into 12 genera, specifically using the seed-coat anatomy. Currently, only 4 species have been retained in *Thlaspi* s.str and all of the other species were divided into other genera, one of which was *Thlaspiceras* F.K.Mey.. Based on recent molecular studies and morphological features, Al-Shehbaz (2014) suggested recognizing all of Meyer's segregates under *Noccaea* and *Thlaspi* s.str. All of Meyer's *Thlaspiceras* species, namely *Th. dolichocarpum* (Zohary) F.K.Mey., *Th. eigii* (Zohary) F.K.Mey., *Th. crassifolium* A.Huber-Morath and F.K.Mey., *Th. bovis* F.K.Mey., *Th. oxyceras* (Boiss.) F.K.Mey., *Th. capricornutum* F.K.Mey., *Th. huber-morathii* F.K.Mey., *Th. cappadocicum* (Boiss. and Balansa) F.K.Mey., *Th. triangulare* F.K.Mey., *Th. rechingeri* F.K.Mey., and *Th. elegans* (Boissier) F.K.Mey., grow in Turkey or on the Syrian side of the border between Turkey and Syria. Meyer classified *Thlaspiceras* taxa under 3 sections: *Dolichocarpa* F.K.Mey., *Thlaspiceras*, and *Acornuta* F.K.Mey.. The current study makes use of the above mentioned species, except for *Th. oxyceras*, *Th. capricornutum* F.K.Mey., *Th. dolichocarpum* (Zohary) F.K.Mey., and *Th. bovis* F.K.Mey. The plant materials used in this research were collected in Turkey between 2014 and 2016. Specimens were identified according to Meyer's identification and named according to Meyer. A total of 12 inter-simple sequence repeat (ISSR) primers, with 52 reliable polymorphic bands, were used in the cluster analyses. The dendrogram obtained supported all of the previously proposed classifications. A detailed discussion regarding previous classifications of the group and the clusters revealed using the ISSR markers is also provided herein.

Key words: Inter-simple sequence repeat, taxonomy, *Noccaea*, *Thlaspiceras*, Turkey

1. Introduction

The systematics of the genus *Noccaea* Moench was one of the most controversial among the 351 *Brassicaceae* genera (Al-Shehbaz, 1986; Al-Shehbaz, 2014; Koch et al., 2018). Until Meyer's revisions (1973, 1979), members of the genus were categorized in the genus *Thlaspi* L., which was represented by approximately 75 taxa that grow mainly in Eurasia (Al-Shehbaz, 1986; Al-Shehbaz, 2014; Appel and Al-Shehbaz, 2003). Meyer (1973, 1979) separated *Thlaspi* into 11 additional genera and listed only 6 species in the *Thlaspi* s.str. *Camelineae*. However, Esmailbegi et al. (2018) showed that 2 species, namely *Thlaspi alliaceum* Linnaeus (646: 1753) and *T. oliveri* Engler (223: 1891) of *Thlaspi* s.str. are separated from the remaining 4 *Thlaspi* s.str. species, and they placed these 2 species under genus *Mummenhoffia* Esmailbegi and Al-Shehbaz, which was introduced as a new genus.

Many taxonomical studies have taken place on the generic delimitation of *Noccaea*, since debates regarding the systematics of the genus have made it very attractive for researchers (Al-Shehbaz et al., 2006; Al-Shehbaz, 2012; Al-

Shehbaz, 2014; Clapham, 1964; Engler and Prantl, 1891; Fırat et al., 2014; Koch and Mummenhoff, 2001; Hedge, 1965; Mummenhoff and Zunk, 1991; Mummenhoff and Koch, 1994; Mummenhoff et al., 1997; Schulz, 1936; Zunk et al., 1996). Among these studies, researchers who used morphology to delimitate the generic (as well as species) circumscription of *Noccaea* members mainly used floral features (i.e. leaves and fruit shapes). In addition to these taxonomical features, Meyer (1973, 1979) noticed that seed-coat anatomy was conservative, and he mainly used this feature for generic delimitation. However, his approach was disapproved by some taxonomists (Al-Shehbaz, 1986; Greuter et al., 1986; Greuter and Raus, 1983) and rejected because of its impractical usage and unnatural taxonomical system (Al-Shehbaz, 2014; Aytaç et al., 2006).

DNA-based studies have also showed that Meyer's concept was artificial (Mummenhoff et al., 1997; Koch and Mummenhoff, 2001), and the only morphology-based classification was problematic due to the convergence of the fruit features. Based on these molecular and morphological studies, Al-Shehbaz (2014) suggested the recognition of 10

* Correspondence: btarikah@hacettepe.edu.tr

segregates by Meyer under the genus *Noccaea*, which is in the monogeneric tribe *Coluteocarpeae sensu* Al-Shehbaz. The remaining segregates were assigned under *Thlaspi* s.str (in the tribe *Thlaspideae*) and *Noccidium* F.K.Mey. (in the tribe *Camelineae*). However, it was corrected as *Coluteocarpeae* in German (2018) and also shown that *Noccidium* is actually nested in tribe *Coluteocarpeae* (Özüdoğru et al., 2019). Phylogenetic analysis of Özüdoğru et al. (2019) indicated generic delimitation of *Noccaea*, which Al-Shehbaz proposed was more acceptable. However, the debate is still ongoing and, according to an alternative point of view that was presented in Brassibase (Koch et al., 2018), the tribe *Coluteocarpeae* consists of 13 different genera, most of which were proposed by Meyer (1973). Among the genera Meyer proposed, *Thlaspiceras* is a confusing genus in terms of detecting the species' boundaries (Özüdoğru, 2018). Following the generic circumscription of Al-Shehbaz (2014), Özüdoğru (2018) investigated *Thlaspiceras* as a species complex under *Noccaea sensu* Al-Shehbaz.

Thlaspiceras species complex consists of 11 species, 9 of which are endemic to Turkey. Only 2 members, *Thlaspi elegans* Boiss. and *T. oxyceras* Boiss., of this complex were included by Hedge in the first volume of Flora of Turkey (1965). Nine additional species of *Thlaspiceras* species complex were added to the supplementary volume of Flora of Turkey (Davis et al., 1988). Some of these species were once evaluated under *T. oxyceras*, but recent studies (Meyer, 2003; Özüdoğru, 2018) have shown that this species is restricted to the Amanos mountain range.

Meyer (2003) classified 11 *Thlaspiceras* taxa into 3 series: *Dolichocarpa* F.K.Mey., *Thlaspiceras* F.K.Mey., and *Acornuta* F.K.Mey. Having a small horn on the fruit and long silicula are the characteristic features of the series *Dolichocarpa*. Members of the series *Thlaspiceras* have distinct and recognizable horns on their fruits and, in the series *Acornuta*, the wings of the fruit are stretched out like a horn, but they can easily be distinguished by their obtuse wings, while a horn is acuminate in all of the other series. However, Al-Shehbaz (2014) proposed that these features were quantitative and not reliable. Although Meyer (2003) claimed that misunderstanding the border of the commissures between the wings and horns could result in false identification, he insisted on using these characteristics to classify the members of the genus.

Molecular-based systematic study on taxa of *Thlaspiceras* species complex was performed by Özüdoğru (2018). He showed that the genus is not monophyletic and the members of the genus group together according to their distribution pattern, contrary to their phylogenetic relationship. Moreover, in a study by Özüdoğru et al. (2019), the phylogeny tree reconstruction of *Noccaea* consisted of the *Thlaspiceras* species, and this research

confirmed the merging of the *Thlaspiceras* taxa under *Noccaea* that Al-Shehbaz (2012, 2014) had proposed.

To develop additional data for solving taxonomical problems, inter-simple sequence repeat (ISSR) variations were used to investigate the genetic relationships within *Thlaspiceras* in this study. This technique was chosen since it is practical and useful in categorizing species level (Reddy et al., 2002). The aim of this study was to make a contribution to the taxonomy of this group of species using the ISSR technique.

2. Materials and methods

2.1. Plant materials

Plant materials were collected during field excursions in Turkey between 2014 and 2016. A detailed locality list of the specimens used in this study is given in Table 1. Young fresh leaves from 1 to 5 plants per species/population were collected during the excursions and stored in silica gel. The plant specimens were identified using Al-Shehbaz's identification key (Al-Shehbaz, 2014) and placed under the genus *Noccaea* in this study. *Noccaea oxyceras* and *Noccaea capricornutum* grow very close to the Turkish border with Syria; hence, it was not possible to collect these species. Although 3 field trips took place to the type localities of *Noccaea dolichocarpa*, it was not possible to find specimens with features that fit the description provided by Meyer (2003).

2.2. DNA Isolation and polymerase chain reaction

DNA isolation, polymerase chain reaction (PCR) and agarose gel electrophoresis were performed according to the protocols explained by Yaman et al. (2014) as follows: The DNA was isolated by using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNA quality was checked in a 1% agarose gel. PCR amplifications were done according to Qiagen's protocol with some modifications: in a total volume of 25 µL master mix (PCR Master Mix Fermentas) containing 20–50 ng of genomic DNA, 1 µM primer, and 1 unit of Taq DNA polymerase. PCR reactions were started with an initial denaturation of 2 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 2 min at 52 °C (annealing), and 40 s at 72 °C (extension), with a final extension step at 72 °C for 5 min. Amplified products were separated by 2% agarose gel electrophoresis with 1× TBE buffer and stained with ethidium bromide. DNA bands were visualized using a KODAK GL 200 Imaging Cabinet (Eastman Kodak Company, NY, USA). The ISSR primers (UBC set 9) used herein came from the Biotechnology Laboratory of the University of British Columbia in Canada.

2.3. Data analysis of the ISSR

Of the 73 oligonucleotide primers that were applied, 12 primers formed reliable polymorphic bands (Table 2). Amplified bands were coded as present = 1 and absent =

Table 1. List of investigated taxa and localities.

Species	No.	Locality and collector Information
<i>Noccaea eigii</i> (Zohary) Al-Shehbaz	1	Hatay: Ziyaret RES- Serinyol Yolu, K. Özgüşi 1086
	2	Hatay: Kızıldağ-Kisecik, Maden Barakaları, K. Özgüşi 1062
	3	Hatay: Kızıldağ Maden Barakaları, K. Özgüşi 1057
<i>Noccaea huber-morathii</i> (F.K.Mey.) Al-Shehbaz	4	Erzincan: Refahiye to Erzincan 27.km. B. Özüdoğru 3678
<i>Noccaea bovis</i> (F.K.Mey.) Al-Shehbaz	5	Hatay: Kaledibi village, K. Özgüşi 1084b
<i>Noccaea elegans</i> (Boiss.) Al-Shehbaz	6	Adana: Hamidiye-Büyük Sofulu arası. B. Özüdoğru 3609
	7	Adana: Pozantı, Çetinlik. B. Özüdoğru 3617
<i>Noccaea triangularis</i> (F.K.Mey.) Al-Shehbaz	8	Osmaniye: Zorkun plateau, B. Özüdoğru 3633.
<i>Noccaea rechingeri</i> (F.K.Mey) Al-Shehbaz	9	Hatay: Kaledibi Köyü, K. Özgüşi 1084a
	10	Hatay: Kaledibi Köyü, K. Özgüşi 1084c
	11	Osmaniye: Zorkun-Erzin, B. Özüdoğru 3629.
	12	Hatay: Dörtüyük, K. Özgüşi 1077
	13	Hatay: Kaledibi village, K. Özgüşi 1084a
<i>Noccaea meyeri</i> Al-Shehbaz	14	Osmaniye: Yarpuz –Yağlıpınar arası, B. Özüdoğru 3623
	15	Osmaniye: Koyunmeleten south-we of koyunmel. plateau, B. Özüdoğru 3640.
	16	Osmaniye: Koyunmeleten plateau, B. Özüdoğru 3645
	17	Osmaniye: Zorkun, Karanlı dere, B. Özüdoğru 3632
	18	Osmaniye: Yarpuz village exit, Daz road, B. Özüdoğru 3628

Table 2. List of the ISSR primer sequences, sizes of base pairs that were revealed, and number of polymorphic bands obtained for the 19 accessions of the *Noccaea* taxa.

Primers	Sequence 5' to 3'	Tm (°C)	Size (bp) min-max	Number of polymorphic bands
	AGCTCCTCCTCCTCCTCC	60	250-850	6
	CTGTCCTCCTCCTCCTCC	60	150	1
UBC807	AGAGAGAGAGAGAGAGT	50	100-900	13
UBC808	AGAGAGAGAGAGAGAGC	52.8	200-700	3
UBC817	CACACACACACACACAA	50.4	550-1100	4
UBC819	GTGTGTGTGTGTGTG TA	50.4	900	1
UBC840	GAGAGAGAGAGAGAGAYT	52.5	100-900	5
UBC847	CACACACACACACARC	54.8	300-1200	5
UBC880	GGAGAGGAGAGGAGA	50.6	500-900	3
UBC889	DBDACACACACACACAC	51.2	600-900	2
UBC890	ACGACTACGGTGTGTGTTGTGT	52.0	200-700	7
UBC891	HVHTGTGTGTGTGTGTG	51.2	700-900	2

0 for each of the 12 primers, according to their fragment sizes. The fragment sizes were determined manually using a 100-base pair (bp) Plus DNA Ladder (Fermentas, Carlsbad, CA, USA). A total of 52 reliable bands were used in the analysis. In order to check the reproducibility of

ISSR bands, a control PCR was done with 4 individuals for each primer. For clustering analysis (CA), the numerical taxonomy and multivariate analysis program, written for the IBM PC by Rohlf (2000), was used. The unweighted pair group average (UPGMA) clustering algorithm, with

the Jaccard similarity coefficient, was used for the CA (Rohlf, 2000).

In addition, the genetic similarities matrix from the calculative data was used to construct a dendrogram based on the neighbour-joining method using PAST software (Hammer et al., 2001). Since a similar tree was obtained, bootstrap values (with 1000 replicates) were given on the UPGMA tree.

3. Results

A total of 52 reliable polymorphic bands were revealed using 12 ISSR markers. The ISSR primers used in the analysis are given in Table 2. Only 1 polymorphic band was obtained with the "CTGTCCTCCTCCTCCTCC" primer, while 13 polymorphic bands were obtained with the UBC807 primer. The average frequency of the bands per primer was 4.33. The band length ranged from approximately 100 bp to 1200 bp.

The UPGMA clustering algorithm grouped species from different series together. Two members of *Dolichocarpa* series, *N. eigii* and *N. bovis*, nested together in a clade with an other species, *N. meyeri*, of this series, clustering together and forming a clade separately. Instead of *N. meyeri*, *N. huber-morathii*, which was evaluated under the *Thlaspiceras* series by Meyer (2003), nested in the *N. eigii* and *N. bovis* clade (Figure).

Members of the *Acornuta* series, *N. rechingeri* and *N. triangularis*, grouped together but another member of this series, *Noccaea elegans*, joined the clade consisting of *N. eigii*, *N. bovis*, and *N. huber-morathii*. Although other members of the *Acornuta* series, namely *N. rechingeri* and *N. triangularis*, formed a separate cluster, *N. elegans* clustered with other series' members. *N. huber-morathii* tend to group with members of *Dolichocarpa* although specimens of *N. eigii* formed a distinct group, which was remote from other members of the *Dolichocarpa* series. The group containing *N. meyeri* specimens separated from the cluster, which other specimens nested in, with a low coefficient value (0.45). Between the members of same series, bootstrap values and coefficient values were relatively high at more than 0.50 (Figure).

According to Al-Shehbaz's identification key (Al-Shehbaz, 2014), fruit with small horns (up to 0.5 mm) and truncate apex are diagnostic characters for *Noccaea eigii*. Specimens collected from Ziyaret RES and Kızıldağ (Hatay) were assigned under *N. eigii* because of their fruit features (Figure). The fruit of specimens from the Kaledibi (Hatay) population have a distinct horn at the apex. This narrowly oblong fruit is characteristic of *N. bovis*. Although oblong fruit is also characteristic of *N. meyeri*, this species has a shorter horn at the apex of the fruit (1.2–1.5 mm for *N. meyeri*; 1.5–2.5 mm for *N. bovis*). The fruits of *N. meyeri* are also wider than those of *N. bovis*. Specimens from

Osmaniye with horned fruit were identified as *N. meyeri*.

Another specimen with horned fruit from Refahiye (Erzincan) was identified as *N. huber-morathii*. Besides a shorter horn at the fruit apex, an obdeltoid and narrower fruit are also characteristic features used to separate this species from *N. bovis* (Al-Shehbaz, 2014).

Fruit shape was used by Meyer (2003) to specify the boundaries of series. According to Meyer's classification, specimens with a short (4–7.5 mm), obdeltoid and horned fruit belong to the *Thlaspiceras* series, whereas specimens with long (7–5 mm), oblong, and horned fruit belong to the *Dolichocarpa* series. Of the species used in this study, only *N. huber-morathii* belongs to the *Thlaspiceras* series. Other specimens, namely *N. eigii*, *N. bovis*, and *N. meyeri* with horned fruit, belong to the *Dolichocarpa* series, according to Meyer's delimitation (2003).

Winged fruit without a horn or a bulge resembling a horn are characteristic of Meyer's *Thlaspiceras* sect. *Acornuta* series. This series consist of 3 different species: *Noccaea elegans*, *N. rechingeri*, and *N. triangularis*. Within these taxa, *N. triangularis* morphologically differs from the other taxa by its triangular fruit, whereas *N. elegans* and *N. rechingeri* have ovate or oblong fruit. *N. elegans* differs from *N. rechingeri* by its oblong fruit (*N. rechingeri* has ovate fruit) and shorter petals (*N. elegans* = 5 mm; *N. rechingeri* = 3.4 mm). Only specimens from Zorkun (Osmaniye) were identified as *N. triangularis*, and specimens from Adana, lacking a horn on the silicle, belong to *N. elegans*, according to Al-Shehbaz's identification key (Al-Shehbaz, 2014).

4. Discussion

During the field excursions, huge variation was observed, especially in the fruit characteristics of different populations, even within the same species. Morphology-based classification could result in error, considering the convergent evolution in fruit morphology, which has mainly been used for the classification of a genus.

Molecular DNA studies, such as the ISSR method, could bring new systematic insight (Yaman et al., 2014; Tarıkahya-Hacıoğlu, 2016). Therefore, it was decided herein to use the ISSR technique to examine the taxonomic relations within the genus. The data obtained from the ISSR markers revealed 2 major clusters. The first cluster consisted of only *Noccaea meyeri* specimens, which were proposed under the series *Dolichocarpa* by Meyer. On the other hand, *N. bovis* and *N. eigii*, which are other species in the series *Dolichocarpa*, were grouped with taxa from Meyer's section *Thlaspiceras*. Zohary (1941) claimed that *N. eigii* should be placed under the section *Carpoceras* DC., whose members have wingless silicula with prominent horns at the apex. However, Greuter et al. (1986) proposed the placement of *Th. eigii* under *Th. elegans* aggr. This

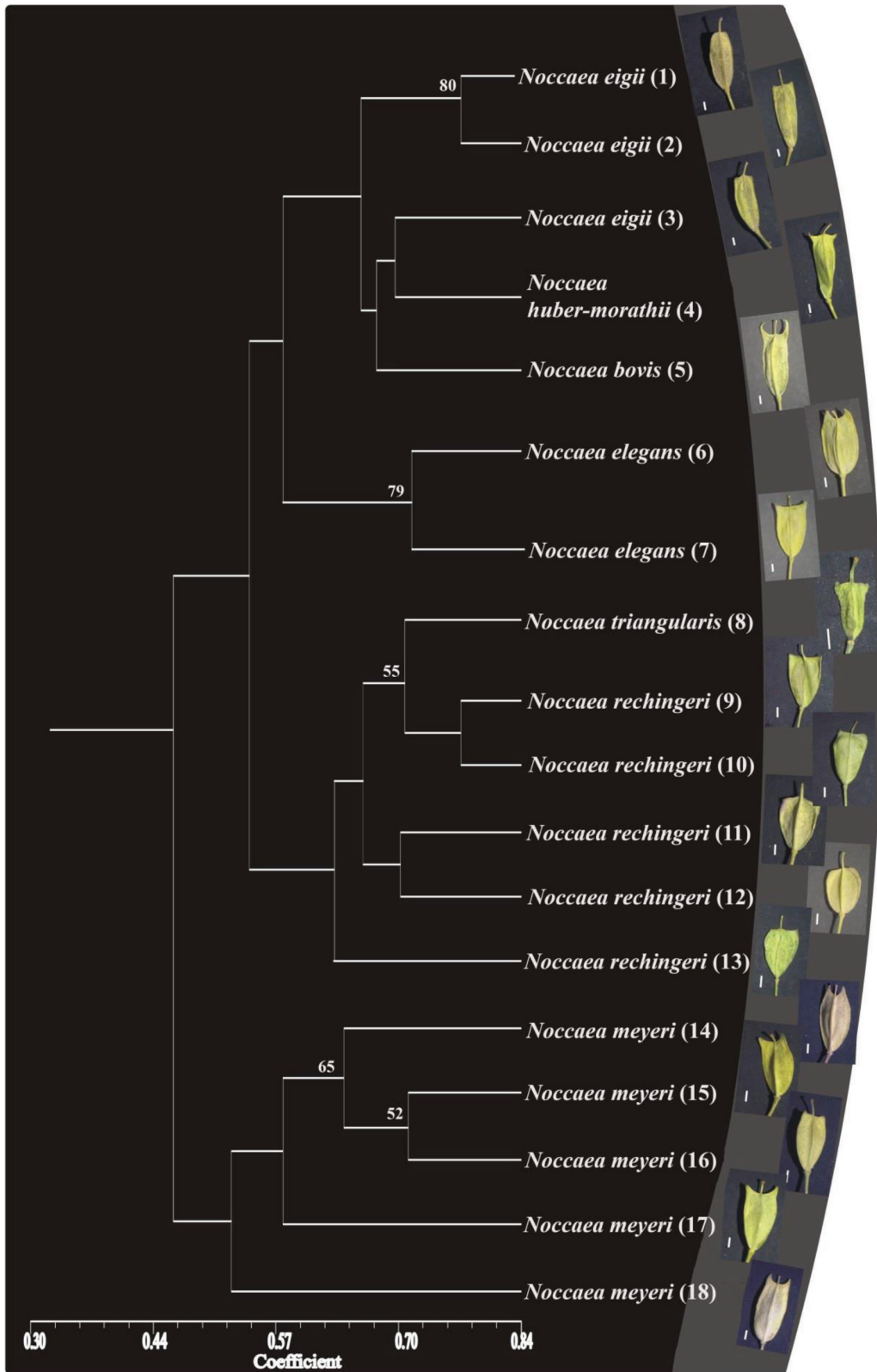


Figure. ISSR dendrogram of the studied *Noccaea* species constructed with UPGMA and fruit photographs of the specimens (scale bars in the photos = 1 mm). Values above the branches show bootstrap percentages (only the bootstrap percentages larger than 50% were indicated).

aggregate is represented by *N. elegans* (Boiss.) Al-Shehbaz, which is a member of *Thlaspi* Sect. *Pterotropis* DC., whose members have obcordate silicula with wings at the apex or above the middle section. Hedge (1965) also proposed that delimitations, which were only dependent on the absence (*Thlaspi* Sect. *Pterotropis*) or presence (*Thlaspi* Sect. *Carpoceras*) of well-developed horns at the apex of the silicula, were rather artificial.

The current research grouped the species with winged silicula as Meyer (2003) had originally claimed. When the detailed descriptions that were given by both Boissier (1867) and Meyer (2003) were carefully studied, the character combinations used to distinguish *N. elegans* and *N. rechingeri* were sepal–petal length and shape of the fruit (*N. elegans* have oblong silicula and 5-mm petals; *N. rechingeri* have ovate silicula and 3.4-mm petals). However, the examined specimens and the distribution patterns of these taxa showed that there was great variation in the fruit and flower features. On the Amanos mountain range, these taxa nested closely together and had no distinct geographical border between them. Although winged fruit seem to be a reliable character for this cluster, curved wings could also be regarded as another recognizable feature. However, as with the other *Brassicaceae* taxa, in order to make an accurate identification, mature silicles need to be observed.

All of the *N. meyeri* species were nested together as a clade. The basic character to distinguish the members of the section *Dolichocarpa* from the others was the length to width ratio of the fruit. According to our observations during the field trips, the mature silicula was more than 2 times longer than broad; however, the young silicula had variations. As Hedge (1965) previously mentioned, it is often essential to see both flowering and completely mature fruiting material for identification.

According to the phylogeny reconstruction of the *Noccaea* species (Özudoğru et al., 2019) *N. bovis*, *N. eigii*, and *N. huber-morathii* were clustered together. However,

in the mentioned research, the internal transcribed spacer (ITS) data revealed a polytomy for this group. The same polytomy was revealed in the *N. elegans*-*N. bovis* clade and *N. triangulare*-*N. rechingeri*-*N. oxyceras* group. *Noccaea eigii* was revealed as a separate clade. The data supported the *N. cappadocicum*-*N. huber-morathii* cluster and *N. triangulare*-*N. rechingeri* cluster when compared to the ITS phylogeny tree.

In conclusion, the cluster obtained from the ISSR dendrogram showed a concordance with fruit morphology, and the dendrogram supported the previously proposed classifications overall. All of the taxa, except for *Noccaea huber-morathii* from Erzincan, were collected from the southernmost corner of the Amanos Mountain Range (in the subclade *N. eigii*, *N. huber-morathii*). These specimens were located near the hypothetic Anatolian diagonal of Davis (1965). Meyer claimed that the *Thlaspiceras* species, which were distributed in a narrow district, should have evolved by isolation of the species, which have a wide range of distribution (Meyer, 2003). The cluster of *N. eigii* and *N. huber-morathii*, with a 70% coefficient value, could prove Meyer's claim. In the *N. elegans* and *N. rechingeri* clade, confusion in the subgroups of the populations of these 2 species was revealed. During the field excursions, great variations were observed in the diagnostic characters used for the morphological identification of these species. Therefore, the taxonomical status of these species could be revised in detail in future work. We believe full genome sequencing seems to be more promising for generating comprehensive phylogenies for the whole genus and the *Thlaspiceras* species, as proposed by Özudoğru et al. (2019).

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