Research Article

Structural characterization of ITS2 and CBC species concept applications in the tribe Coluteocarpeae (Brassicaceae)

Kurtuluş ÖZGIŞI*

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

Abstract: The taxonomic utility of internal transcribed spacer 2 (ITS2) secondary structures in different plant groups, as well as in Brassicaceae, has been addressed by many studies. Although characterization and applications of ITS2 secondary structures for the members of main Brassicaceae lineages (Lineages I, II, III, and expanded Lineage II) have been studied, the utility of compensatory base change (CBC) species concept has not been the subject of any studies thus far. In the current study, the ITS2 secondary structures of 49 Coluteocarpeae (expanded Lineage II) specimens were investigated to determine relationships among the species. In addition to the utility of the CBC species concept, the availability of hemi-CBC and nonstructural substitutions (NSTs), which are also used for generic and species delimitation, were tested and discussed. A maximum likelihood tree, based on the sequence-structural alignment of the 49 specimens, was constructed to test the different generic assumptions reported by different researchers in the literature. The structural analysis showed that the ITS2 secondary structures of all of the Coluteocarpeae members exhibited a 4-fingered hand model, which was common in the majority of the family members. No CBCs were observed, whereas hemi-CBCs and NSTs were common among the tribe members. Although hemi-CBCs and NSTs were useful for distinguishing most of the Coluteocarpeae species (*Noccaea aptera* (Velen.) F.K. Mey., *Noccaea aghrica* (P.H. Davis & Kit Tan) M. Fırat & Özüdoğru, *Noccaea fendleri* (A.Gray) Holub subsp. *glauca* (A. Nelson) Al-Shehbaz & M. Koch, *Noccaea griffithiana* (Boiss.) F.K. Mey., *Noccaea rubescens* (Schott & Kotschy ex Boiss.) F.K. Mey., etc.), they were not effective for delimitating some problematic species, such as members of *Thlaspiceras* F.K. Mey. A phylogenetic tree based on the sequence-structural dataset of the ITS2 showed that generic delimitation of Al-Shehbaz was more acceptable due to the fact that *Noccaea* sensu Al-Shehbaz is monophyletic.

Key words: Coluteocarpeae, Brassicaceae, *Noccaea*, secondary structure, ITS2.

1. Introduction

The tribe Coluteocarpeae is the most taxonomically complex group among the 52 Brassicaceae tribes in terms of generic delimitation of the tribe members (https://brassibase.cos.uni-heidelberg.de/, accessed 27 August 2019). Almost all members of the tribe were once subsumed under the genus *Thlaspi* L. Schulz (1936) placed members of *Thlaspi* under the subtribe Thlaspidinae, which was one of the 13 subtribes of the tribe Lepidieae. The main feature used by Schulz (1936) to draw the circumscriptions of the tribe was the presence of angustiseptate fruit. However, it is well known that angustiseptate fruit has evolved independently many times within the family (Mummenhoff et al., 1997a). Many molecular studies (Mummenhoff and Hurka, 1995; Mummenhoff et al., 2001, 2004) have been performed to clarify the tribal boundaries of the family. These studies showed that the assumptions of the Schulz (1936) were artificial, and using this character for classification resulted in unrelated genera

(i.e. *Aethionema* W.T. Aiton, *Isatis* L., *Thlaspi* L., *Iberis* L.) being grouped together under the tribe Lepidieae.

Meyer, influenced by Schulz (1936), insisted on assigning *Thlaspi* and 11 additional genera under the subtribe Thlaspidinae (Meyer, 2001). Meyer (1973, 1979) mainly relied on the seed coat anatomy as one of the most conservative features to classify the members of these genera. Aside from its impractical usage, his approach was considered an unnatural taxonomical system (Al-Shehbaz, 2014; Aytaç et al., 2006) and many researchers refused to accept it (Greuter and Raus, 1983; Greuter et al., 1986; Artelari, 2002). Because of these differences of opinion between the researchers, both the generic and tribal classification of *Thlaspi* remained unclear. However, subsequent studies (Al-Shehbaz et al., 2006; German et al., 2009; Couvreur et al., 2009; Warwick et al., 2010; Al-Shehbaz, 2012) revealed a more accurate tribal classification of the family, indicating that the tribal circumscriptions of both Schulz (1936) and Meyer (2001) were unnatural.

* Correspondence: kurtulusozgisi@gmail.com

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Based on various molecular studies (Koch et al., 2003; Koch and Al-Shehbaz, 2004), Al-Shehbaz et al. (2006) introduced the tribe Noccaeeae Al-Shehbaz, Beilstein & E.A. Kellogg, and Al-Shehbaz (2014) assigned Meyer's (1973; 1979) segregates into this tribe, except for *Noccidium* F.K. Mey. (in the tribe Camelineae) and *Thlaspi* s.str. (in the tribe Thlaspideae). After this, it was shown that *Coluteocarpus reticulatus* Boiss. was nested within *Noccaea* (Warwick et al., 2010; Firat et al., 2014). Since *Coluteocarpus* was grouped with *Noccaea*, Al-Shehbaz (2012) reduced the tribe Noccaeeae to the synonymy of the tribe Coluteocarpeae (expanded Lineage II), which was previously reported by Dorofeyev (2004), and transferred the type species of *Coluteocarpus* Boiss., *C. reticulatus* to *Noccaea* (Al-Shehbaz, 2014).

The tribal classification of Al-Shehbaz (2012) was accepted by some researchers (Ali et al., 2016a; German, 2017; Özgişi et al., 2018a; Özgişi et al., 2018b; https://brassibase.cos.uni-heidelberg.de/, accessed 27 August 2019). However, the generic classification of Coluteocarpeae remains controversial. While some researchers (Özgişi et al., 2018a; Özgişi et al., 2018b; Güzel et al., 2018; Özüdoğru, 2018) have accepted the concept of Al-Shehbaz (2014), others (Ali et al., 2016a; 2016b; Karaismailoğlu and Erol, 2018; 2019; https://brassibase. cos.uni-heidelberg.de/, accessed 27 August 2019) have chosen to follow different concepts.

The internal transcribed spacers (ITSs) of ribosomal RNA have been frequently used molecular markers for the tribal and generic classification of the tribe Coluteocarpeae (Mummenhoff and Zunk, 1991; Mummenhoff et al., 1997b; Koch and Al-Shehbaz, 2004; Warwick, 2010; Özüdoğru et al., 2019). Furthermore, ITSs are the most commonly used (Doğan et al., 2011; Mutlu, 2018; Özgişi, 2020) nuclear markers for estimating phylogenetic relationships among the rest of the family due to their ubiquitous presence, sufficient synapomorphic characters, and cost-effectiveness. In addition to these criteria, the structural characteristics of ITS2, which is 1 of 7 components of the ribosomal RNA gene cluster (Wheeler and Honeycutt, 1988), have been used as a molecular tool for defining the boundaries of a species over the last decade. Although ITS regions can have a high mutation rate, due to point mutations and indels, ITS2 forms a highly conserved secondary structure to catalyse the maturation of the ribosomal RNA (Mai and Coleman, 1997; Morgan and Blair, 1998; Venema and Tollervey, 1999). The 4-fingered structure, UGGU motif near the apex of Helix III (longest helix), and U-U mismatch in Helix II are considered the characteristics of a conserved ITS2 secondary structure (Schultz et al., 2005). Although ITS2 forms a highly conserved secondary structure in the eukaryotes, nucleotide differences in the helices, such as compensatory base changes (CBCs), which are nucleotide changes at both sides of the paired bases, and the length polymorphism of ITS2 are widely used to infer the boundaries of the species (Coleman, 2003; Schultz, 2005; Coleman, 2009; Keller et al., 2010; Wolf, 2013). An experimental study by Coleman and Vacquier (2002) showed that there was a correlation between the CBCs and interspecies sexual compatibility. Taxa differing by the CBCs (even by just 1 CBC) in the conserved pairing positions of the ITS2 secondary structure were experimentally observed to be completely incapable of intercrossing (Coleman and Vacquier, 2002). Since this finding referred to the biological species concept, CBCs have been used as a species delimitation tool by many researchers (Müller et al., 2007; Mullineux and Hausner, 2009; Budak et al., 2016; Saha et al., 2017; Karpenko et al., 2018). Furthermore, CBCs in the ITS2 secondary structure and hemi-CBCs, which involve changes on only 1 side of the nucleotide pair, have also been used to assess relationships at the population and species level (Torres-Suárez, 2014). Moreover, the utility of nonstructural substitutions (NSTs) for determining the boundaries of a species was reported by Karpenko et al. (2018).

In addition to the debates (Greuter and Raus, 1983; Greuter et al., 1986; Artelari, 2002; Aytaç et al., 2006; Al-Shehbaz, 2014; Ali et al., 2016a; German, 2017; Özgişi et al., 2018a; Özgişi et al., 2018b; Karaismailoğlu and Erol, 2018) regarding the generic concept by Meyer (1973; 1979), variations among the morphological features that were used for species delimitation resulted in misevaluation of the species (Özgişi, 2018a). Because of these variations, the morphology-based keys to identify specimens, which was proposed by Meyer (1973; 1979; 2006) and Al-Shehbaz (2014) have been inadequate in some cases (i.e. for distinguishing *N. densiflora* (Boiss. & Kotschy) F.K. Mey., *N. amani* (Post) F.K. Mey., *N. microstyla* (Boiss.) F.K. Mey., and *N. violascens* (Schott & Kotschy) F.K. Mey. from each other) to identify the specimens.

Herein, the secondary structure properties of ITS2 were tested for the tribe Coluteocarpeae, whose generic delimitation has been debated. Although the ITS1 and ITS2 secondary structures of Brassicaceae members, belonging to different lineages (Linages I, II, and III, and expanded Lineage II), were the subject of a previous study (Edgar et al., 2014), the CBC species concept has not yet been tested for the family members. Since the morphological variations cause mistakes when identifying the species, the aim herein was to test if the CBC species concept, as well as hemi-CBCs and NSTs, are useful for distinguishing tribe members. Furthermore, a maximum likelihood (ML) tree, based on the sequence and structural dataset of the ITS2 alignment, was constructed to draw the generic

circumscription of the tribe and the generic delimitations of different researchers were discussed.

2. Materials and methods

2.1. Plant samples

Leaf materials of 49 specimens were obtained from the field or different herbaria (HUB, MO, and HUMZ) and identified in accordance with the keys and descriptions that were proposed by Meyer (1973; 1979; 2006) and Al-Shehbaz (2014). Detailed information about the material is given Table 1. The ITSs of these materials were sequenced. The ITS2 sequences of *Thlaspi arvense* L., which were used in the analysis, were collected from Genbank (http://www.ncbi.nlm.nih.gov/genbank). The Genbank numbers of the investigated species are given in the phylogenetic tree.

2.2. DNA extraction, amplification, and sequencing

Total genomic DNA was isolated using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The ITS region was amplified using primers ITS1 and ITS4 (White et al., 1990). Amplification of the ITSs was performed following the protocol of Warwick et al. (2004). Purification and sequencing were performed by MedSanTek (İstanbul, Turkey).

2.3. Inference of the secondary structure

To estimate the secondary structure of the ITS2 sequences, each sequence was annotated using the hidden Markov model (HMM)-based annotation tool present in the ITS2 database V (Ankenbrand et al., 2015). To define the boarders of the ITS2 of the investigated specimens, the E-value <0.01 and Viridiplantae HMMs with ITS2 minimum-sized >150 nt options, which were suggested by Ankenbrand et al. (2015), were used. Delimited ITS2 sequences from the alignment between the 5.8S and 28S were submitted to the RNA folding program on the Mfold webserver (Zuker, 2003) and ITS2 secondary structures were predicted using a linear sequence, with RNA version 2.3 energy rules and folding temperature set to 37 °C. The ITS2 database, a resource for annotation, ITS2 motif search, secondary structure prediction, and estimating phylogenetic relationships of ITS2 sequences (Koetschan et al., 2012), were also used to predict the secondary structure of ITS2. In the ITS2 database, secondary structures were obtained via energy minimization, which was proposed by Mathews et al. (1999). The obtained structures and sequences of ITS2 were synchronously aligned by 4SALE (Seibel et al., 2006) using a specified 12 × 12 scoring matrix (Wolf et al., 2014) in locally implemented ClustalW (Larkin et al., 2007). The alignment file (with structures) is provided as supplemental information. For better visualization, VARNA 3.9 (Darty et al., 2009) software was used to redraw and annotate the secondary structures of ITS2.

2.4. Phylogenetic analyses and mapping of apomorphic CBCs, hemi-CBCs, and NSTs

The dataset, which contained the ITS2 sequence and structural alignment of 49 specimens, was implemented into the statistical framework R (R core team, 2014) to obtain a ML tree using the phangorn program (Schliep, 2011). *T. arvense*, from the tribe Thlaspideae, was used as an outgroup and the robustness of the ML tree was tested using 1000 bootstrap replicates.

PAUP 4.0b10 (Swofford, 2002) was used to find synapomorphic changes in the clades (or branches) within the tree. The aligned sequences and ML tree were imported into the program. The maximum parsimony optimality criterion (parsimony settings; character state optimization: DELTRAN) was selected and the log file option was activated. All apomorphies (Trees: Describe Trees with phylogram, labeled internal nodes, and list of apomorphies) were obtained and apomorphic traits (CBCs, hemi-CBCs, and NSTs) were labelled by colour on the helices of ITS2 (Figure 1). In addition to labelling of the apomorphic traits by colour, branching of the phylogenetic tree was enumerated and these numbers were indicated on the labelled helices of ITS2 (Figures 1 and 2).

3. Results

3.1. ITS2 secondary structure properties of the tribe Coluteocarpeae

The ITS2 secondary structures of 49 specimens were modelled. The ITS2 regions had variable lengths, a mean length of 187 bp (max = 191 bp; min = 185 bp), and a mean of 2.75 possible secondary structures (Table 2). Approximately 55.67% of all of the nucleotide positions were paired. The GC content of the ITS2 sequences ranged from 49.20% to 53.72%, with an average of 51.50%. All of the Coluteocarpeae members had a 4-fingered ITS2 structure. Their consensus structures are shown in Figure 3. The ITS2 motif search indicated that all of the species had a U-U mismatch (5′-AACUGGUCUCCCGUG, left; 5′-CGCGGUUGGCCAAAA, right) in Helix II. Furthermore, a highly conserved UGGU motif (5′-GACAUGCGGUGGUGA) was detected near the apex of Helix III (Figure 3). Helix III was the longest (usually 83 bp), whereas Helix IV was the shortest (ranging from 14 bp to 17 bp). In some species, Helix IV looped between the 172nd and 186th bases (Figure 4), whereas some only had a terminal loop between the 170th and 180th bases. Estimated thermodynamic energy values of the predicted secondary structures ranged from –68.60 kcal/mol to –57.00 kcal/mol.

3.2. Relationship among the Coluteocarpeae species

The phylogenetic tree constructed from the ITS2 secondary structure alignments is presented in Figure 2. The ML analysis showed that *Eunomia* DC., *Callothlaspi*

Table 1. Collection information of materials used in the analysis. Taxon names followed the concept presented in BrassiBase (https://brassibase.cos.uniheidelberg.de/).

Taxa	Locality, collector, and herbaria information
Callothlaspi camlikense (Aytaç, Nordt & Parolly) F.K. Mey.	Turkey, Konya, Çamlık Village, *Ozudogru 3575*, HUB
C. cariense (Carlström) F.K. Mey.	Turkey, Muğla, Marmaris, *K.O 1034*, HUB
C. lilacinum (Boiss. & A.Huet) F.K. Mey.	Turkey, Erzincan, Refahiye, *Ozudogru 3660*, HUB
Coluteocarpus vesicaria (L.) Holmboe	Turkey, Erzincan, Spikor pass, *Ozudogru 4498* HUB
Eunomia iberidea Boiss.	Turkey, Çankırı, Eldivan Mountain, *Ozudogru 3941*, HUB
E. oppositifolia (Pers.) DC.	Turkey, Nigde, Aladağlar, *Ozudogru 3727,* HUB
Kotschyella cilicica (Schott & Kotschy ex Boiss.) F.K. Mey.	Turkey, Kahramanmaraş, Küçükçamurlu Village *Ozudogru 3657*, HUB
Microthlaspi natolicum (Boiss.) F.K. Mey.	Turkey, Hatay, Yayladağ, *Ozudogru 4025*, HUB
M. perfoliatum (L.) F.K. Mey.	Turkey, Kırklareli, Dereköy, *K.O 1129*, HUB
Masmenia rosularis (Boiss. & Balansa) F.K. Mey.	Turkey, Hatay, Kızıldağ, *K.O 1027*, HUB
Neurotropis platycarpa (Fisch. & C.A. Mey.) F.K. Mey.	Turkey, Hatay, Dörtyol, *K.O 1081*, HUB
Noccaea aghrica (P.H. Davis & Kit Tan) Fırat & Özüdöğru	Turkey, Ağrı, *Firat 30170*, HUB
N. amani (Post) F.K. Mey.	Turkey, Hatay, Dörtyol, *K.O 1078a*, HUB
N. aptera (Velen.) F.K. Mey.	Turkey, Kırklareli, Dereköy, *K.O 1130*, HUB
N. caerulescens (J. Presl & C. Presl) F.K. Mey.	*T.C. 742*, Czech Republic
N. densiflora (Boiss. & Kotschy) F.K. Mey.	Turkey, Kahramanmaras, Kaman Mountain *Ozudogru 3650,*HUB
N. edinensium F.K. Mey.	Turkey, Kütahya, Gediz, *K.O 1087*, HUB
N. fendleri subsp. *glauca* (A. Nelson) Al-Shehbaz & M.A. Koch	USA, *Werff 21551*, MO
N. griffithiana (Boiss.) F.K. Mey.	Pakistan, *Websten & Sack 5661*, MO
N. haussknechtii (Boiss.) F.K. Mey.	Turkey, Kahramanmaraş, Berit Mountain *Ozudogru 4718*, HUB
N. leblebicii (Gemici & Görk) Raus	Turkey, Muğla, Fethiye, *Ozudogru 3808*, HUB
N. microstyla (Boiss.) F.K. Mey.	Turkey, Osmaniye, Düldül Mountain *Ozudogru 4442*, HUB
N. ochroleuca (Boiss. & Heldr.) F.K. Mey.	Turkey, Isparta, Davraz Mountain, *Ozudogru 5009&Ozgisi*, HUB
N. papillosa (Boiss.) F.K. Mey.	Turkey, Antalya, Gazipaşa, *Ozudogru 3597*, HUB
N. phrygia (Bornm.) F.K. Mey.	Turkey, Bolu, Kartalkaya, *K.O 1106*, HUB
N. rubescens (Boiss.) F.K. Mey.	Turkey, Niğde, Aladağlar, *Ozudogru 3734*, HUB
N. sintenisii (Bornm.) F.K. Mey.	Turkey, Bayburt, Karakaya Mountain, *Ozudogru 3694*, HUB
N. tatianae (Bordz.) F.K. Mey.	Turkey, between Erzincan and Gümüşhane *Ozudogru 3681*, HUB
N. valerianoides (Rech.f.) F.K. Mey.	Turkey, Van, Bahçesaray, *Firat 32576*, HUB
N. violascens (Schott & Kotschy) F.K. Mey.	Turkey, Sivas, Şarkışla, *Ozudogru 4470*, HUB
Noccidium hastulatum (DC.) F.K. Mey.	Iran, *A. Naqinezhad,* HUMZ
Pseudosempervivum aucheri (Boiss.) Pobed.	Turkey, Konya, Aydos Mountain, *Erik 1897*, HUB
P. sempervivum (Boiss. & Balansa) Pobed.	Turkey, Osmaniye, Koyunmeleten Mountain *Ozudogru 3610, Ozgisi & Acici,* HUB
P. sintenisii (Hausskn. ex Bornm.) Pobed.	Turkey, Rize, Çamlıhemşin, *Guner 6812*, HUB
Raparia bulbosa (Spruner ex Boiss.) F.K. Mey.	Greece, *Koch 92-001*, HEID
Syrenopsis stylosa Jaub. & Spach	Turkey, Kütahya, Gediz, *K.O 1048*, HUB

Table 1. (Continued).

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Thlaspiceras bovis F.K. Mey.	Turkey, Hatay, Kızıldağ, *K.O 1058*, HUB
T. cappadocicum (Boiss. & Balansa) F.K. Mey.	Turkey, Sivas, Arslandoğmuş Village, *Ozudogru 4479*, HUB
T. crassifolium Hub.-Mor. & F.K. Mey.	Turkey, Osmaniye, Zorkun, *Ozudogru 3632*, HUB
T. eigii (Zohary) F.K. Mey.	Turkey, Hatay, Samandağ, *K.O 1086*, HUB
T. elegans (Boiss.) F.K. Mey.	Turkey, Adana, Pozantı, *Ozudogru 3609*, HUB
T. huber-morathii F.K. Mey.	Turkey, Erzincan, Refahiye, *Ozudogru 4486,* HUB
T. rechingeri F.K. Mey.	Turkey, Osmaniye, Zorkun, *Ozudogru 3629*, HUB
T. triangulare F.K. Mey.	Turkey, Hatay, Serinyol, *K.O 1085*, HUB
Vania campylophylla F.K. Mey.	Turkey, Van, Güzeldere, *Fırat 30608*, HUB
V. kurdica (Hedge) F.K. Mey.	Turkey, Van, Ispiriz Mountain, *Fırat 31009*, HUB
V. pulvinata F.K. Mey.	Turkey, Van, Ispiriz Mountain, *Fırat 31036*, HUB
V. trinervia (DC.) Khosravi et al.	Turkey, Van, Nebruz Plateau, *Fırat 30671*, HUB
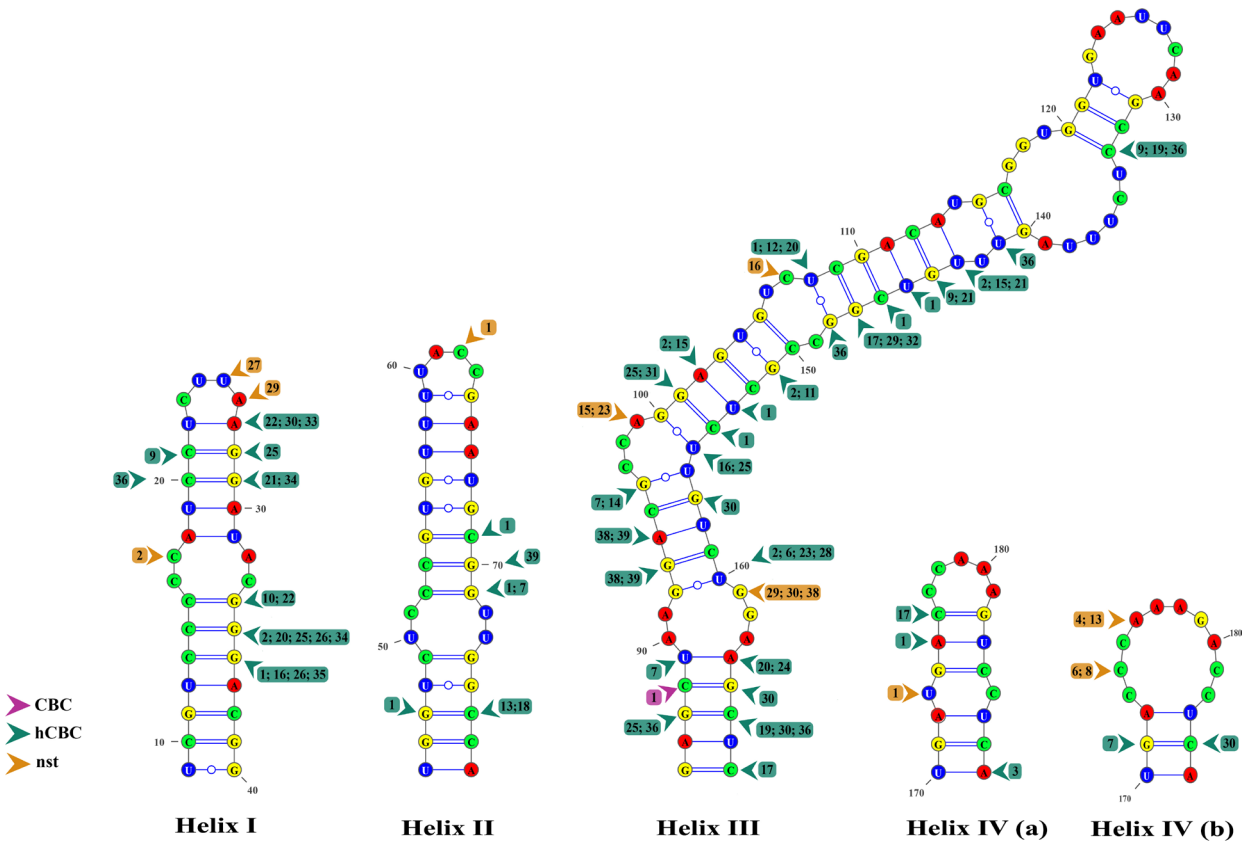
Figure 1. Compensatory base changes (CBCs), hemi-CBCs (hCBCs), and nonstructural substitutions (NSTs) on the helices of ITS2 in the tribe Coluteocarpeae. Two types of Helix IV are presented. Helix IV (**a**) loops between the 172nd–174th and 184th–186th bases, whereas Helix IV (**b**) has only 1 terminal loop. The CBCs are marked in violet, the hemi-CBCs in green, and the NSTs in orange. Numbers in the coloured circle indicate the branching points that were shown on the phylogenetic tree.

F.K. Mey., *Noccaea* sensu Meyer, and *Vania* F.K. Mey. were clearly polyphyletic.

There was only 1 CBC detected in Helix I, which occurred between the outgroup and the rest of the specimens. Hemi-CBCs and NSTs were more common than CBCs among the tribe members and outgroup. In total, 10 hemi-CBCs and 2 NSTs (1 hemi-CBC in Helix I; 3 hemi-CBCs and 1 NST in Helix II; 5 hemi-CBCs in
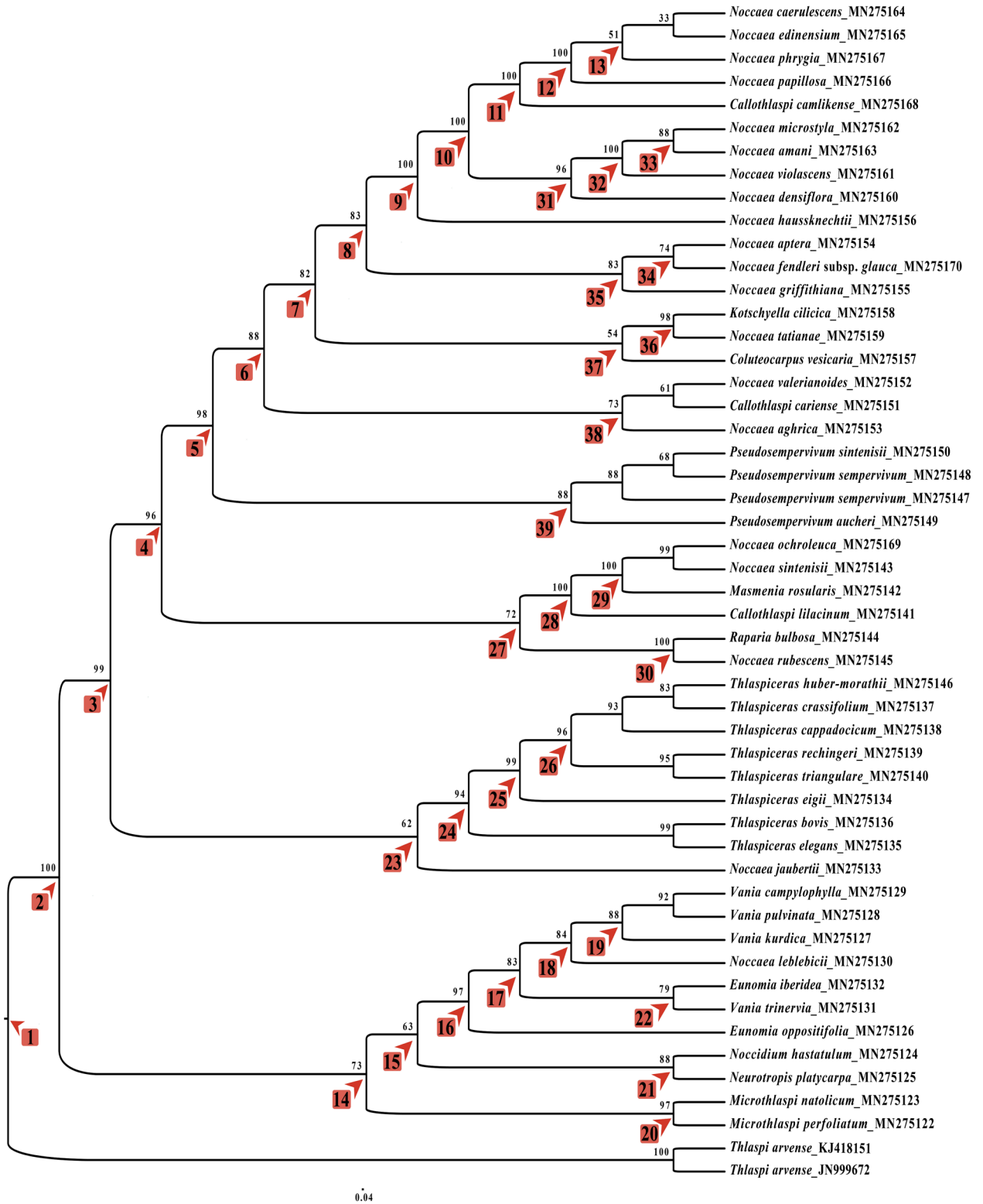
Figure 2. Phylogenetic tree obtained with the ML approach using the structural alignment data of ITS2 in the tribe Coluteocarpeae. Species were named by following Brassibase (https://brassibase.cos.uni-heidelberg.de/, accessed 27 August 2019). *T. arvense* (in the tribe Thlaspideae) was used as an outgroup and the bootstrap values are shown on the nodes. Branching points were enumerated and shown on the nodes.

Table 2. Characteristics of the sequence and secondary structure of ITS2 in members of Coluteocarpeae (T_m: proportion of ΔH (structure enthalpy) and ΔS (structure entropy) to determine the folding properties. ΔG: minimum free energy level for folding). Taxon names followed the concept presented in BrassiBase (https://brassibase.cos.uniheidelberg.de/).

Species and GenBank accession number	Length (nt)	GC content	T_m °C	ΔG kcal/mol	Helix lengths (nt)			
					Helix I	Helix II	Helix III	Helix IV
*Callothlaspi camlikense*_MN275168	188	53.72	78.7	−64.50	31	34	83	16
*C. cariense*_MN275151	188	52.66	79.6	−61.70	31	34	83	16
*C. lilacinum*_MN275141	188	50.53	77.8	−57.00	31	34	83	17
*Coluteocarpus vesicaria*_MN275157	188	52.13	79.4	−59.10	31	34	83	16
*E. iberidea*_MN275132	188	51.06	78.5	−60.80	31	34	83	17
*E. oppositifolia*_MN275126	188	51.06	78.5	−60.80	31	34	83	17
*Kotschyella cilicica*_MN275158	188	51.60	78.9	−58.20	31	34	83	16
*Microthlaspi natolicum*_MN275123	190	54.45	79.5	−68.50	34	34	83	17
*M. perfoliatum*_MN275122	191	50.00	81.4	−68.60	34	34	82	17
*Masmenia rosularis*_MN275142	188	55.26	78.5	−58.00	31	34	83	17
*Neurotropis platycarpa*_MN275125	187	52.13	78.0	−58.70	31	36	83	17
*Noccaea aghrica*_MN275153	188	49.20	79.6	−60.50	31	34	83	16
*N. amani*_MN275163	188	51.60	78.0	−58.80	31	34	83	16
*N. aptera*_MN275154	188	51.60	81.5	−63.70	31	34	83	16
*N. caerulescens*_MN275164	188	52.13	78.7	−61.50	31	34	83	16
*N. densiflora*_MN275160	188	53.19	78.0	−56.20	31	34	83	16
*N. edinensium*_MN275165	188	50.53	78.7	−61.50	31	34	83	16
N. fendleri subsp. *glauca*_MN275170	188	53.19	79.0	−61.20	31	34	83	16
*N. griffithiana*_MN275155	188	53.19	78.8	−60.00	31	34	83	16
*N. haussknechtii*_MN275156	187	53.16	82.0	−66.10	33	35	83	16
*N. leblebicii*_MN275130	188	51.06	78.5	−60.80	31	34	83	17
*N. microstyla*_MN275162	188	51.06	75.6	−53.90	31	34	83	16
*N. ochroleuca*_MN275169	189	50.53	79.2	−59.50	31	34	83	17
*N. papillosa*_MN275166	184	52.66	77.6	−59.30	31	34	83	16
*N. phrygia*_MN275167	188	53.72	78.8	−63.50	31	34	83	16
*N. rubescens*_MN275145	188	50.53	79.5	−62.10	31	34	83	17
*N. sintenisii*_MN275143	188	50.00	79.2	−59.50	31	34	83	17
*N. tatianae*_MN275159	188	52.66	75.6	−53.10	31	34	83	16
*N. valerianoides*_MN275152	188	51.60	79.6	−61.70	31	34	83	16
*N. violascens*_MN275161	188	49.73	78.2	−58.80	31	34	83	16
*Noccidium hastulatum*_MN275124	183	52.66	78.6	−54.60	31	34	83	14
*Pseudosempervivum aucheri*_MN275149	185	51.35	79.9	−61.50	31	34	83	15
*P. sempervivum*_MN275147	188	52.41	79.6	−61.70	31	34	83	16
*P. sempervivum*_MN275148	187	52.66	79.6	−61.70	31	34	83	16
*P. sintenisii*_MN275150	188	52.13	79.5	−61.60	31	34	83	16
*Raparia bulbosa*_MN275144	188	50.53	77.2	−56.30	31	34	83	17
*Syrenopsis stylosa*_MN275133	188	51.34	80.0	−61.10	31	34	82	17
*Thlaspiceras bovis*_MN275136	188	50.53	80.4	−60.10	31	34	83	17

Table 2. (Continued).

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*T. cappadocicum*_MN275138	188	50.54	79.7	−61.10	31	34	83	16
*T. crassifolium*_MN275137	188	50.53	80.1	−61.60	31	34	83	17
*T. eigii*_MN275134	188	50.53	80.1	−60.70	31	34	83	17
*T. elegans*_MN275135	188	50.53	80.4	−60.10	31	34	83	17
*T. hubermorathii*_MN275146	188	50.53	80.1	−61.60	31	34	83	17
*T. rechingeri*_MN275139	188	50.53	79.9	−61.60	31	34	83	17
*T. triangulare*_MN275140	188	50.53	79.9	−61.60	31	34	83	17
*Vania campylophylla*_MN275129	188	50.00	78.3	−60.80	31	34	83	17
*V. kurdica*_MN275127	187	49.20	78.5	−60.80	31	34	83	17
*V. pulvinata*_MN275128	188	50.00	78.3	−60.80	31	34	83	17
*V. trinervia*_MN275131	188	51.06	78.5	−60.80	31	34	83	17
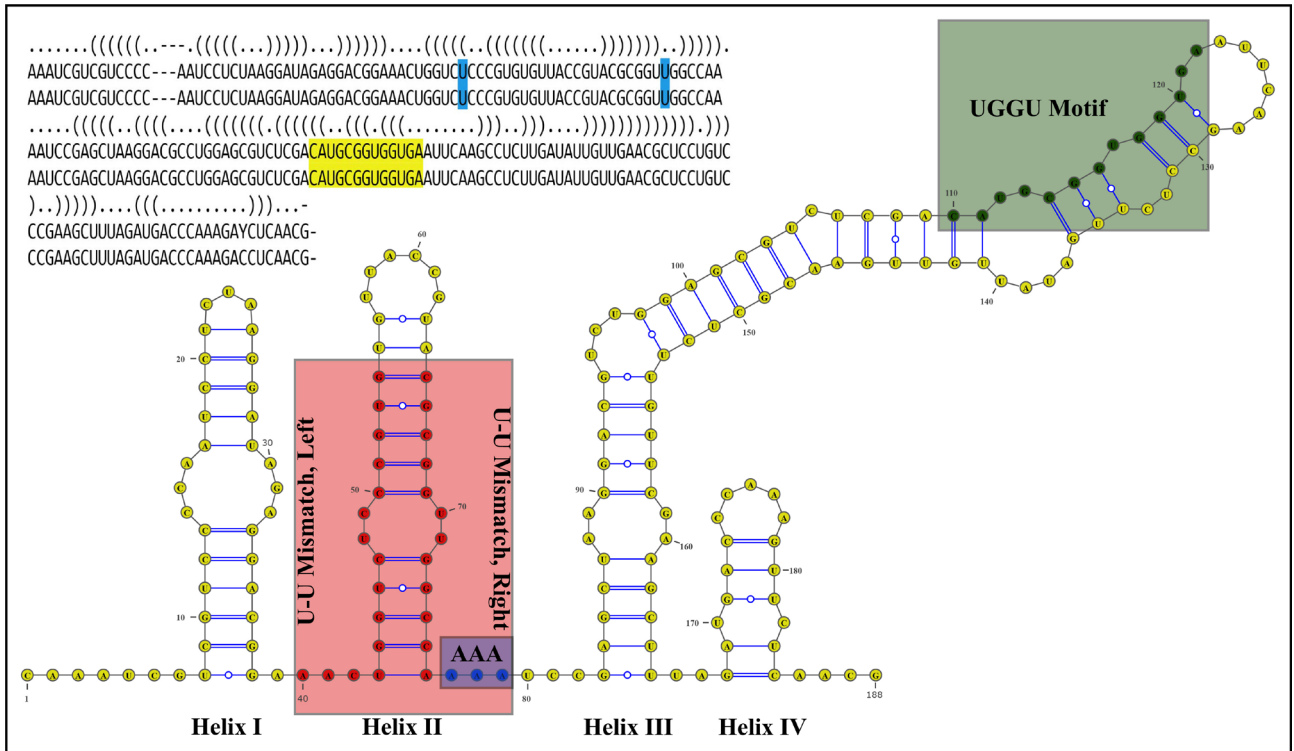
Figure 3. The U-U mismatch (Helix II, left; Helix II, right), AAA (between Helix II and III), and UGGU motif (at 5′ to the apex of Helix III) are the major ITS2 motifs for plants. Pairwise sequence alignment based on a universally conserved secondary structure of both sequences (*N. rubescens*_MG944863 and *N. valerianoides*_MG944864) is also given. The U-U mismatches are highlighted in blue and the UGGGU motifs are highlighted in yellow.

Helix III, and 1 CBC and 1 NST in Helix IV) (Figure 3) were detected as apomorphic features, which caused the differentiation on node 1. Hemi-CBC and NST differences among the Coluteocarpeae taxa are presented in Table 3.

Although *Noccaea edinensium* F.K. Mey. and *Noccaea caerulescens* (J.Presl & C.Presl) F.K. Mey. were different from each other morphologically, there were no hemi-CBCs or NSTs detected among these species (Figure 2). Similarly, there was no difference between some of the species, i.e. *Noccaea valerianoides* (Rech.f.) F.K. Mey. and *Callothlaspi cariense* (Carlström) F.K. Mey.; *Pseudosempervivum sintenisii* (Hausskn.) Pobed. and *Pseudosempervivum sempervivum* (Boiss. & Balansa) Pobed., in terms of CBCs, hemi-CBCs, and NSTs. Bootstrap values on these nodes were relatively
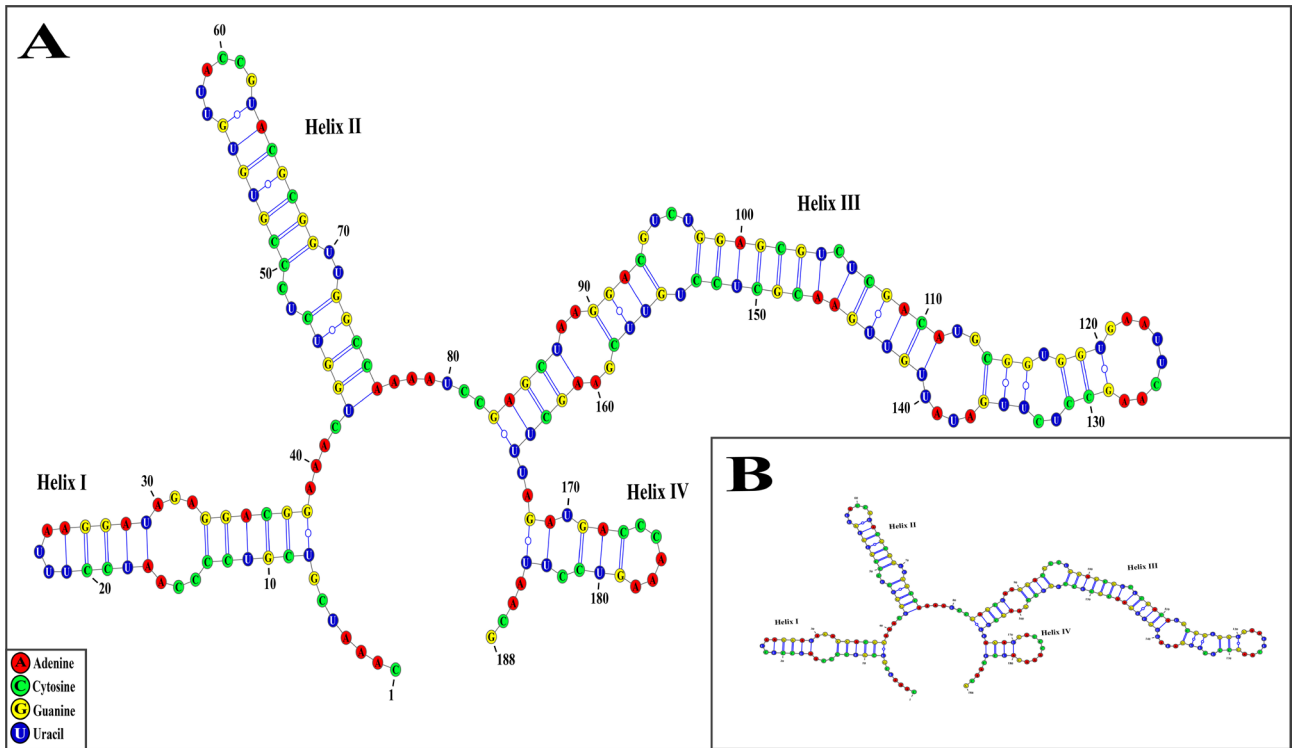
Figure 4. Common ITS2 secondary structure model of tribe Coluteocarpeae **A.** Looped Helix IV **B.** Helix IV with 1 terminal loop.

low (Figure 2). Despite high bootstrap values and different morphologies, no hemi-CBCs were observed between some other species, i.e. *Noccaea sintenisii* (Hausskn. ex Bornm.) F.K. Mey. and *Noccaea ochroleuca* (Boiss. & Heldr.) F.K. Mey.; and some *Pseudosempervivum* (Boiss.) Grossh. and *Thlaspiceras* F.K. Mey. species (Figure 2).

4. Discussion
ITS has been frequently used as a marker for estimation the relationships among Brassicaceae members (German et al., 2009; Khosravi et al., 2009; Warwick et al., 2010). Edger et al. (2014) indicated that solely using ITS1 and ITS2 is insufficient for delimitation taxonomic groups due to the lack of sufficient signals; however, some studies (Müller et al., 2007; Coleman, 2009) have reported that there was a positive correlation between the presence of a CBC in the ITS2 secondary structure and sexual incompatibility. This assumption referred to the biological species concept. Moreover, the utility of hemi-CBCs and NSTs to distinguish species has been reported in some studies (Torres-Suárez, 2014; Karpenko et al., 2018).

In the present study, these concepts (CBCs; hemi-CBCs, and NSTs) were tested for the tribe Coluteocarpeae. Although some species distinctly differed from each other morphologically, the ITS2 dataset showed that there was only 1 CBC (in Helix I) between the outgroup and the rest of the species. However, lacking a CBC between tribe

members does not mean that these organisms are the same species. Müller et al. (2007) reported that a CBC occurring between different genera was more common, since species belonging to same genus are highly related to each other. Torres-Suárez (2014) tested the utility of the CBC species concept and determined that CBCs can be used for generic delimitation as well. On the basis of Torres-Suárez (2014) findings, only one CBC, between the outgroup and the tribe Coluteocarpeae, indicates that *Noccaea* sensu Al-Shehbaz (2014) was more acceptable despite of some studies (Ali et al., 2016a; 2016b; Karaismailoğlu and Erol, 2018; 2019; https://brassibase.cos.uni-heidelberg.de/, accessed 27 August 2019) which disagree with generic delimitation of Al-Shehbaz (2014). This finding is also supported by previous studies (German, 2017; Özgişi, 2018a; Özgişi et al., 2018b; Güzel et al., 2018; Özüdoğru and German, 2018; Özüdoğru et al., 2019) which consider that *Noccaea* sensu Al-Shehbaz (2014) is more acceptable.

Moreover, the structural analysis (with ITS2 sequences) clearly supported both the tribal and generic circumscriptions of Özüdoğru et al. (2019), who followed the concept of Al-Shehbaz (2014). Özüdoğru et al. (2019) reported that the genus *Noccidium* F.K. Mey. was nested within the tribe Coluteocarpeae (previously it was placed in the tribe Camelineae). The phylogenetic analysis of this study also indicated that *N. hastulatum* was nested within the tribe Coluteocarpeae. However, the CBC species

Table 3. Hemi-CBC and NST differences between the Coluteocarpeae members. Numbers indicate the position of changed nucleotide(s).

Taxa	Hemi-CBC	NST
Callothlaspi camlikense (Aytaç, Nordt & Parolly) F.K. Mey.	151	
C. lilacinum (Boiss. & A.Huet) F.K. Mey.	160	
Coluteocarpus vesicaria (L.) Holmboe		169
Eunomia iberidea Boiss.	27, 34	
E. oppositifolia (Pers.) DC.	36	107, 155
Kotschyella cilicica (Schott & Kotschy ex Boiss.) F.K. Mey.	87, 133, 141, 148, 166	
Microthlaspi natolicum (Boiss.) F.K. Mey.	35, 108, 164	
M. perfoliatum (L.) F.K. Mey.	35, 108, 164	
Masmenia rosularis (Boiss. & Balansa) F.K. Mey.	147	26, 161
Neurotropis platycarpa (Fisch. & C.A. Mey.) F.K. Mey.	29, 143, 144	
Noccaea aghrica (P.H. Davis & Kit Tan) Fırat & Özüdoğru	93, 94	161
N. amani (Post) F.K. Mey.	27	
N. aptera (Velen.) F.K. Mey.	29, 35	
N. densiflora (Boiss. & Kotschy) F.K. Mey.	101	
N. fendleri subsp. *glauca* (A. Nelson) Al-Shehbaz & M.A. Koch	29, 35	
N. griffithiana (Boiss.) F.K. Mey.	36	
N. haussknechtii (Boiss.) F.K. Mey.	21, 144	
N. leblebicii (Gemici & Görk) Raus.	76	
Noccaea microstyla (Boiss.) F.K. Mey.	27	
N. papillosa (Boiss.) F.K. Mey.	108	
N. phrygia (Bornm.) F.K. Mey.	76	176
N. rubescens (Boiss.) F.K. Mey.	27, 157, 165, 166, 184	161
N. tatianae (Bordz.) F.K. Mey.	87, 133, 141, 148, 166	
N. violascens (Schott & Kotschy) F.K. Mey.	157	
Noccidium hastulatum (DC.) F. K. Mey.	29, 143, 144	
Pseudosempervivum aucheri (Boiss.) Pobed.	70, 93, 94,	
Raparia bulbosa (Spruner ex Boiss.) F.K. Mey.	27, 157, 165, 166, 184	161
Syrenopsis stylosa Jaub. & Spach	160	
Thlaspiceras eigii (Zohary) F.K. Mey.	35, 87, 101, 155	
Vania kurdica (Hedge) F.K. Mey.	133, 166	
V. trinervia (DC.) Khosravi et al.	27,34	

concept was inadequate for distinguishing this species from its relatives.

Hemi-CBCs and NSTs were more common than CBCs among the tribe members and outgroup. Although Torres-Suárez (2014) used hemi-CBCs to distinguish different species, there were no hemi-CBCs or NSTs detected between some of the Coluteocarpeae species. For instance, despite the fact that *Noccaea edinensium* and *N. caerulescens* morphologically resemble one another, there were no hemi-CBCs observed between these species.

Furthermore, the phylogenetic tree, obtained from the sequence structure synchronous analysis, showed that the bootstrap value of this node was very low; therefore, ITSs do not seem to be a suitable marker for distinguishing these species (Figure 2). The absence of hemi-CBCs could indicate a lack of sufficient evolutionary distance between these species. Even though some nodes had high bootstrap values, no hemi-CBCs were detected on these nodes. For instance, although the ITS2 structure analysis showed that there was no difference between *N. sintenisii* and *N.*

ochroleuca, the DNA sequence (bootstrap value of 99) and morphological data revealed these were different species. Same patterns were observed between *Pseudosempervivum* species as well. The phylogenetic study of Özüdoğru et al. (2019) reported that *P. sempervivum* (Boiss. & Balansa) Pobed. and *P. sintenisii* (Hausskn. ex Bornm.) Pobed were clearly different from each other. Although all *Pseudosempervivum* species were assigned under the genus *Noccaea* s.l. by Özüdoğru et al. (2019), herein, the species was named according to the Brassibase data, to show the complications with the determinations used on Brassibase. However, there were no hemi-CBCs or NSTs observed between these species. These findings showed that solely using the hemi-CBC concept is an insufficient tool to distinguish Coluteocarpeae species, at least for some species.

Members of the tribe Coluteocarpeae share a common ITS2 secondary structure. This 4-fingered structure model is common among Brassicaceae (Edgar et al., 2014). Although there was at least 1 hemi-CBC or NST among the helices, there was a conserved region in Helix III. Aside from its role in the cleavage, this site is also considered as a protein binding site (Müller et al., 2007). The only difference in the structure among the tribe members occurred in Helix IV. The length polymorphism and structural difference of Helix IV were considered the result of RNA strand slippage events occurring in Helix III and are common among most eukaryotes (Mullineux and Hausner, 2009; Budak et al., 2016). Since it has a common shape, the ITS2 secondary structure is not a useful tool to distinguish tribe members.

On the basis of this study, it was shown that the CBC species concept was inadequate for the delimitation of tribe members, whereas hemi-CBCs seemed to be more useful. However, the absence of hemi-CBCs among some morphologically different species indicated that solely using hemi-CBCs was also inadequate for the delimitation of Coluteocarpeae species. Moreover, the lack of CBCs, hemi-CBCs, or NSTs did not mean that this concept was completely ineffective for the tribe Coluteocarpeae. Müller et al. (2007) indicated that a lack of CBCs did not mean that these organisms were the same species. On the basis of this assumption, well-annotated ITS2 sequences and the CBC concept (with hemi-CBCs and NSTs) can be used together to distinguish most Coluteocarpeae members when further data (morphology, or other markers etc.) are also used.

Acknowledgements

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