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Centaurea konkae and *C. appendicata* (Asteraceae, Magnoliophyta): features of ITS1 and ITS2 sequences secondary structure

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Summary: The results of comparative morphological analysis and analysis of ITS1-ITS2 rDNA secondary structure are provided for two rare species, *Centaurea konkae* and *C. appendicata* (section *Pseudophalolepis*), which are critically endangered and Ukrainian endemics (Lower Dnieper region). *C. konkae* and *C. appendicata* differ from each other and from closely related species of this section, including *C. donetzica* and *C. breviceps* based on morphological characteristics. This pair of species is an intermediate group between molecular micro-clades 'Ukraine 1' (*C. donetzica* and *C. protogerberi*) and 'Ukraine 2' (*C. breviceps*, *C. margaritacea*, *C. margarita-alba*, *C. protomargaritacea*) of Eastern ribotype Centaurea group on secondary structure ITS1 and ITS2. The fact that *C. appendicata* and *C. konkae* possess single nucleotide polymorphism (SNP) in sites ITS1 (67.Y, 134.R) and ITS2 (21.Y, 41.Y), which alternative alleles differentiate micro-clades 'Ukraine 1' and 'Ukraine 2', allows to assume that these species originated as a result of hybridization of representatives of micro-clade 'Ukraine 1' (*C. donetzica*) and micro-clade 'Ukraine 2' (*C. breviceps*) with further allopatric division of the initial hybrid population into two species: *C. appendicata* on the right bank of the Dnieper River and *C. konkae* on the left bank of the Dnieper River.

Keywords: Appendicatae, *Centaurea,* endemic species, ITS1, ITS2, *Pseudophalolepis*, Ukraine

A number of *Centaurea* L. species, which are narrow regional endemics, has been described from the territory of Ukraine. Among those are *Centaurea konkae* Klokov and *C. appendicata* Klokov which are considered to be local endemic species of the lower reaches of the Dnieper and included in the 'Red Data Book of Ukraine' (Didukh & Akimov 2009). The relevance of research of pearl knapweeds *C. konkae* and *C. appendicata* is of immediate interest because of threatening condition of populations of these species. For instance, *C. appendicata* is now represented by a single population that consists of less than 300 plants and is threatened with extinction less than in next ten years (e.g. over the past two years, the population decreased more than 20%). The other species, *C. konkae*, is represented by two populations that consist of 800–1000 specimens (Moysiyenko et al. 2014a). *C. konkae* and *C. appendicata* are together with other Ukrainian endemic species part of pearl knapweeds taxonomic aggregate of the subgenus *Phalolepis* (Cass.) Dobrocz. and section *Pseudophalolepis* Klokov (Dobrochaeva 1965).

According to the famous Ukrainian botanist M.V. Klokov, the section *Pseudophalolepis* consists of 10 endemic species of *Centaurea*, which grow in the Ukrainian steppe zone (Klokov 1935). These species represent a paleopontic group of old Mediterranean species and form four so-called 'rows': *Pseudoalbae* Dobrocz. (*C. pseudoleucolepis* Kleopow), *Eumargaritaceae* Klokov (*C. protomargaritacea* Klokov, *C. margaritacea* Ten. and *C. margarita-alba* Klokov), *Appendicatae* Klokov (*C. konkae*, *C. appendicata*) and *Gerberianae* Klokov (*C. protogerberi* Klokov, *C. donetzica*

Klokov, *C. breviceps* Iljin, *C. paczoskii* Kotov) (Dobrochaeva 1965). These rare species are listed in the latest edition of 'Red Data Book of Ukraine' (Didukh & Akimov 2009).

All *Pseudophalolepis* sections, except of *C. konkae* and *C. appendicata*, were investigated at the molecular level, in particular, using sequences of internal transcribed spacers 1 and 2 (ITS1 and ITS2) of nuclear rDNA (Garcia-Jacas et al. 2006; Suárez-Santiago et al. 2007; Mráz et al. 2012; Hilpold et al. 2014; Moysiyenko et al. 2014b).

It was shown that the sequences of Ukrainian pearl knapweeds (along with a part of the Crimean species of the section *Phalolepis* (Cass.) DC.) are combined into a so-called 'AP' ribotype (initials of *Acrolophus-Phalolepis* sections) (Suárez-Santiago et al. 2007). Later, the position of Ukrainian pearl knapweeds in the system proposed by Hilpold (Hilpold et al. 2014) was defined more precisely. According to this system, the genus *Centaurea* was separated into three subgenera (*Centaurea* L., *Lopholoma* (Cass.) Dobrocz. and *Cyanus* (Mill.) Hayek). The pearl knapweeds belong to subgenus *Centaurea*. It is divided into three clearly distinguished molecular clades: Eastern Mediterranean Clade (EMC), Western Mediterranean Clade (WMC) and Circum-Mediterranean Clade (CMC). The Ukrainian pearl knapweeds are in the CMC-clade. This clade includes six groups of species allocated on the basis of molecular-cladistics approach: 'Akamantis', 'Ammocyanus', 'Cnicus', 'Hierapolitana', 'Jacea-Phrygia' and the largest 'Centaurea' group including pearl knapweeds. Within 'Centaurea' group, three main ribotypes can be distinguished: a) 'Western ribotype', which in general corresponds to section *Willkommia* and includes the nomenclatural type of the genus *Centaurea*, *C. paniculata* L. (Greuter et al. 2001); b) 'Moroccan ribotype' (combines species known now as row group Simulans); c) 'Eastern ribotype' which corresponds to the group of sections '*Acrolophus-Phalolepis*'as proposed in a previous work (Suárez-Santiago et al. 2007).

The 'Eastern ribotype' group contains 8 micro-clades granted specifying names and several tens of secluded branches, for which the system of phylogenetic relationship within the group has not been resolved yet. Ukrainian pearl knapweeds belong to three micro-clades ('Eastern ribotype: Ukraine 1' (*C. donetzica*, *C. protogerberi*); 'Eastern ribotype: Ukraine 2' (*C. breviceps*, *C. margaritacea*, *C. margarita-alba*, *C. protomargaritacea*); 'Eastern ribotype: Balkan' (*C. paczoskii* together with more than ten species that do not belong to pearl knapweeds) and a separate branch (*C. pseudoleucolepis*). Thus, the Hilpold system (Hilpold et al. 2014) generally coincides with Klokov's (1935) ideas about the geographic 'row' of Ukrainian pearl knapweeds, in particular, with regard to the separateness of *C. pseudoleucolepis* (*Pseudoalbae* 'row'), close ties of *C. protomargaritacea*, *C. margaritacea* and *C. margarita-alba* (*Eumargaritacea* 'row') and *C. protogerberi* with *C. donetzica* (*Gerberianae* 'row'). The differences lie in the removal of two species from *Gerberianae* 'row': *C. breviceps* and *C. paczoskii* (the first, *C. breviceps*, is the representative of the *Eumargaritacea* 'row', as molecular phylogenetic data proved and the other, *C. paczoskii*, is only distantly related to other pearl knapweeds). These conclusions coincide with the analysis of the ITS1 and ITS2 secondary structures of pearl knapweeds, representatives of the 'rows' *Pseudoalbae*, *Eumargaritacea* and *Gerberianae* (Moysiyenko et al. 2014b). However, the degree of validity of the allocation of *C. konkae* and *C. appendicata* into the independent *Appendicatae* 'row' as well as their links with other species of pearl knapweeds remains unclear till now.

Materials and methods

In the present study, we used leaf fragments of four *C. konkae* and *C. appendicata* specimens collected in July and August 2014 in all known localities of these species, including the *locus classicus* (Moysiyenko et al. 2014a). The *C. konkae* samples represent two populations: a) 14 July 2014 (N 47°26'22", E 35°16'51", 82 m) 'Velyki Kuchuhury', island Husiachyi, National Nature Park 'Velykyi Luh', Vasylivka district, Zaporizhya region, Ukraine, *locus classicus*, sample S7; b) 13 August 2014 (N 48°34'36", E 34°37'00", 60 m) suburbs of Kamians'ke, Kurylivka town, Petrykivka district, Dnipropetrovs'k region, Ukraine, sample S3. Both samples of *C. appendicata* (S10 and S11) were collected in *locus classicus*, the single known locality of this species in 13 July 2014 (N 47°39'24", E 35°5'50", 39 m), Lysohirka village, Zaporizhya district, Zaporizhya region, Ukraine. Samples deposited in herbarium KHER (*C. appendicata*: № 10010, 10011; *C. konkae*: № 10012, 10013, 10014, 10015) and KWU (*C. appendicata*: № 59622, 59623; *C. konkae*: № 59624, 59625) were selected for morphological investigation. Fragments of green plants were selected and dried in silica gel for molecular genetic research.

Morphological methods

Morphological research of *C. konkae*, *C. appendicata* and two closely related species (*C. donetzica*, *C. breviceps*), which showed the highest similarity by results of molecular genetic analysis, was performed using light binocular microscope MBS-10 according to common morphological methods on green plants in their habitats and the herbarium samples were deposited in KHER, KW and KWU.

DNA isolation, amplification and sequencing

Total DNA was extracted from silica gel-dried or herbarium specimens by CTAB buffer (Doyle & Doyle 1990) using the modified technique developed for herbarium specimens (Tarieiev et al. 2011), amplified and sequenced using ITS5 and ITS4 universal primers (White et al. 1990). Amplification was performed using a standard technique (Chassot et al. 2001). Purification and sequencing of PCR products were commercially carried out at Macrogen Europe Laboratory (www.macrogen.com Amsterdam, Netherlands). Sequence editing was conducted manually by visual inspection of obtained chromatograms using BioEdit software package (www.mbio.ncsu.edu/bioedit/bioedit.html). Obtained sequences were deposited in NCBI (http://www.ncbi.nlm.nih.gov/) with accession numbers KX950818, KX950819 for *C. appendicata* (samples S11 and S10) and KX950820, KX950821 for *C. konkae* (samples S7 and S3).

Secondary structure models

Annotation of ITS1 and ITS2 sequences was performed by comparison of *C. konkae* and *C. appendicata* sequence regions with earlier annotated *C. breviceps* ITS1 and ITS2 sequences (KJ961606) (Moysiyenko et al. 2014b). ITS1 and ITS2 secondary structure models were constructed by direct transcript formulation in mFOLD (Zuker 2003) and by sequential build of helices according to the model proposed for *C. breviceps* (Moysiyenko et al. 2014b). Obtained models of ITS1 and ITS2 secondary structures were visualized by PseudoViewer (ver. 3.0) (Byun & Kyungsook 2006).

Dataset for comparison was formed from ITS1 and ITS2 sequences which were most similar to *C. konkae* and *C. appendicata* (similarity higher than 99%). The search for such sequences was

Table 1. Morphological differences of stems and leaves between *C. konkae*, *C. appendicata*, *C. donetzica*, *C. breviceps.*

Morphological characters	*C. konkae*	*C. appendicata*	*C. donetzica*	*C. breviceps*
Stem height (cm)	up to 60	up to 90	up to 50	up to 100
Branching of the stem	above the middle of the stem	in the middle of the stem	below the middle of the stem	below the middle of the stem
Stem surface	smooth	rough	rough	rough
Leaf segment width (mm)	2–3.5	1–3	1–2	1–1.5
Leaf surface and its borders	smooth	rough	rough	smooth

performed using NCBI BLAST and a query ITS1–5.8S rDNA–ITS2 sequence of *C. konkae* and *C. appendicata*, MEGABLAST was used as a search algorithm (http://blast.ncbi.nlm.nih.gov).

Results

Morphological research

Centaurea konkae, *C. appendicata*, *C. breviceps* and *C. donetzica* do not differ in morphological characteristics such as the shape of the roots (all solid, vertical), leaves (leaf segments linear), color of achenes and pappus (all light to dark brown, pappus is white). The basic dimensions (height of a stem, size of leaves, involucres, appendages, flowers and achenes) overlap, although they slightly differ in the range of limited values (Tables 1, 2).

Centaurea konkae and *C. appendicata* are well distinguishable from *C. breviceps* and *C. donetzica* by following criteria: rounded-rhombic appendages, present keel and edges on appendages and absent ribs on leaves of involucres.

Centaurea konkae and *C. appendicata* differ from each other in height, branching stems and pubescence, in width of leaves and hairs on their borders, in shape, size and color of involucres and leaves of involucres, in appendages size, color spots on the appendages, color and size of flowers (Figs 1, 2) and in the length of pappus (Table 2).

Figure 1. *Centaurea konkae.*

Figure 2. *Centaurea appendicata.*

Table 2. Morphological differences of involucres, appendages, flowers, achenes and pappus between *C. konkae*, *C. appendicata*, *C. donetzica* and *C. breviceps* (measures in mm).

Morphological characters	*C. konkae*	*C. appendicata*	*C. donetzica*	*C. breviceps*
Involucre shape	subglobose	globose	subglobose	ovoid
Involucre size	15–16 × 16–17	22–27 × 22–27	12–15 × 10–16	10–14 × 6–10
Color of involucre leaves	yellowish, no ribs	greenish, no ribs	pale green with reddish ribs	pale green with green ribs
Middle appendage shape	rounded rhomboid	rounded rhomboid	elliptical	elliptical
Middle appendage size	6–7 × 8–10	10–12 × 6–11	5–6 × 4–4.5	4–5 × 3–5
Spot size on the appendage	oblong-triangular	oblong-triangular	shortly triangular	oblong-triangular
Spot color on the appendage	reddish	dark purple	reddish brown	brownish purple
Keel on appendage	present	present	no	no
Edge on appendage	short, soft	short, soft	no	no
Flower color	pale purple	pale yellow	pale pink	purple
Flower length	14–16	20–25	20–22	10–14
Achene length	3.5–5	4.5–5	4–5	3–4
Pappus length	4.5–5.5	5–7	4–5	2.5–3.5

The morphological characteristics presented by us completely coincide with the diagnostic characteristics which were listed by Klokov (1935) and Dobrochaeva (1965) and confirmed by our investigations.

Thus, *C. konkae* and *C. appendicata* differ from other similar species and from each other on the morphological level.

Molecular genetic studies

For both samples of *C. appendicata* and for the sample of *C. konkae* from *locus classicus*, we obtained 681 bp long sequences, which included complete sequences of ITS1, 5.8S rRNA, ITS2 and partial sequences of 18S rRNA and 28S rDNA (Table 3).

The sequence from *C. konkae* specimen from suburbs of Kamians'ke was 146 bp long and included partial sequences of 18S rDNA and ITS1.

The sequences of both *C. appendicata* samples were identical, the sequences of *C. konkae* were also identical in overlapping regions. The *C. appendicata* sequences had four single nucleotide

Table 3. Length sequences (bp) in *C. konkae* and *C. appendicata* samples.

Species	Sample	total lenght	18S rDNA	ITS1	5.8S rDNA	ITS2	28S rDNA
C. appendicata	l.cl.-S11	681	38 p	255 c	166 c	210 c	12 p
C. appendicata	l.cl.-S10	681	38 p	255 c	166 c	210 c	12 p
C. konkae	l.cl.-S7	681	38 p	255 c	166 c	210 c	12 p
C. konkae	Kam-S3	146	38 p	108 p	-	-	-

Abbreviations: l.cl. – specimens from *locus classicus*; p – partial sequences, c – complete sequences; Kam – specimen from suburbs Kamians'ke.

polymorphism sites (SNP). Five sites with SNP were dedected in *C. konkae*, four of them were the same as in *C. appendicata*. The only difference between *C. konkae* and *C. appendicata* was found. It was site 155 of ITS1 (helix 2), where both samples of *C. appendicata* have cytosine (C), but *C. konkae* has SNP Y (C/T) (Fig. 3).

BLAST (http://blast.ncbi.nlm.nih.gov/) search results showed that *C. appendicata* and *C. konkae* ITS1–5.8S–ITS2 sequences share 99.4% similarity with *C. donetzica* (JF913988, JF913986) and with *C. protogerberi* (DQ319149) and 99.0–99.2% similarity with *C. breviceps* (KJ961607, KJ961606). Therefore, the sequences of these species were selected for comparison of their ITS1 and ITS2 secondary structures with those of *C. konkae* and *C. appendicata*.

The conservative motives of ITS1,2 (helices ITS1 H2, H3, and ITS2 H1, H2, H3) coincided with the patterns of the secondary structure of these sequences proposed for Asteraceae (1C, 1B, 2A, 2B, 2C, respectively). The other helices were specific to the genus *Centaurea* (Goertzen et al. 2003).

ITS1 secondary structure

Obtained models of *C. konkae* and *C. appendicata* ITS1 secondary structure included four main (H1–H4) and two additional (Ha and Hb) helices (Fig. 4).

The only difference detected between these species was the presence of a single nucleotide polymorphism in site 139.Y in the second helix of *C. konkae* instead of site 139.C characteristic for *C. appendicata*, which does not change the secondary structure of the helix in both alleles. Thus, there were no differences in secondary structure models between ITS1 *C. konkae* and *C. appendicata*.

Centaurea konkae and *C. appendicata* differ from related species *C. donetzica, C. protogerberi* and *C. breviceps* by SNP presence in sites 67 and 134. Notably, 67.T and 134.A alleles of *C. appendicata* are identical to ITS1 sequence of *C. donetzica*, while the alternative alleles (67.C and 134.G) are identical to *C. breviceps*. ITS1 secondary structures, however, remain the same in these species. According to the Coleman concept, (Coleman 2000, 2007, 2009), the similarity of secondary structure (in the absence of compensatory base changes) hints at the absence of reproductive isolation between these taxa. The fact that alternative alleles of *C. konkae* and *C. appendicata* match with both with *C. donetzica* and *C. breviceps* ITS1 sequences indicates the intermediate position of species of *Appendicatae* 'row' within the micro-clades listed by Hilpold et al. (2014) as 'Eastern ribotype: Ukraine 1' (*C. donetzica*, *C. protogerberi*) and 'Eastern ribotype:

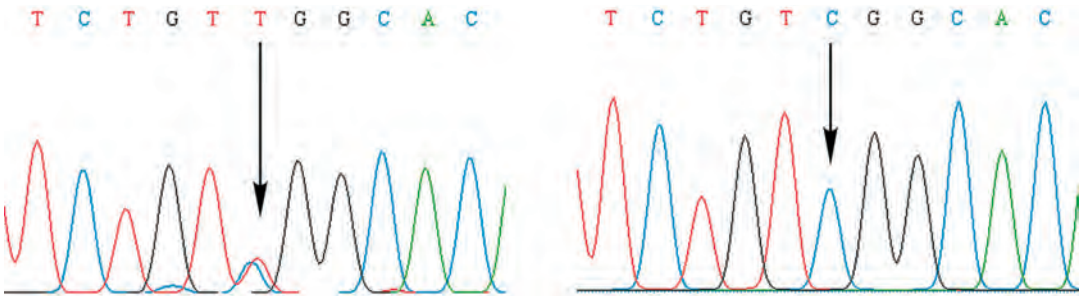

Figure 3. The differences in sequences *C. konkae* (left) and *C. appendicata* (right) in site 155 ITS1 (for example, samples S7 and S10, respectively)

Ukraine 2' (*C. breviceps*, *C. margaritacea*, *C. margarita-alba*, *C. protomargaritacea*). Moreover, *C. donetzica* (JF913988, JF913986) and *C. breviceps* (KJ961606, KJ961607) shared the highest similarity with the relevant alternative alleles of *C. appendicata* (100% similarity) and *C. konkae* (99.61% similarity).

ITS2 secondary structure

Centaurea konkae and *C. appendicata* ITS2 secondary structure models were identical with the ring-model with four helices (Fig. 5).

Both species had two single nucleotide polymorphism sites (sites 21.Y and 41.Y) in the first helix. The presence of SNP in these sites explicitly distinguished *C. konkae* and *C. appendicata* from *C. protogerberi*, *C. donetzica* and *C. breviceps* (in the latter species these sites did not contain SNP). This 21.T + 41.T allele was identical to *C. protogerberi* (DQ319149) and *C. donetzica* (JF913988) ones, while alternative 21.T + 41.T ITS2 allele was identical to *C. breviceps* (KJ961606, KJ961607). Notably, the replacement C > T in sites 21 and 41 seems to be leading to changes in ITS2 first helix secondary structure of *C. donetzica* and *C. protogerberi* on the one side and *C. breviceps*, on the other side. So, *C. konkae* and *C. appendicata* represented an intermediate variant between *C. donetzica* and *C. protogerberi* with respect to their ITS2 secondary structure, similarly to ITS1 (micro-clade 'Eastern ribotype: Ukraine 1') and *C. breviceps* (micro-clade 'Eastern ribotype: Ukraine 2').

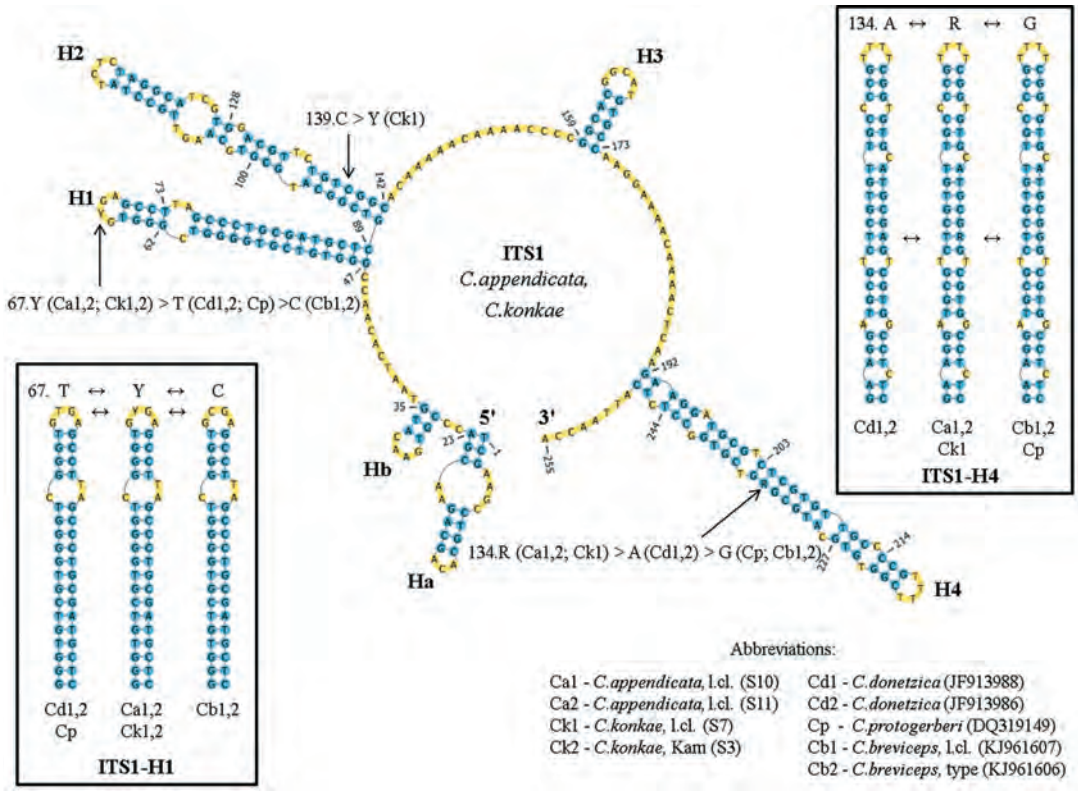

Figure 4. ITS1 secondary structure model of *C. konkae* and *C. appendicata* with variable sites that distinguish it from related species (*C. breviceps*, *C. donetzica* and *C. protogerberi*) and variants of first (ITS1-H1) and fourth (ITS1-H4) helices structures of these species (highlighted frame).

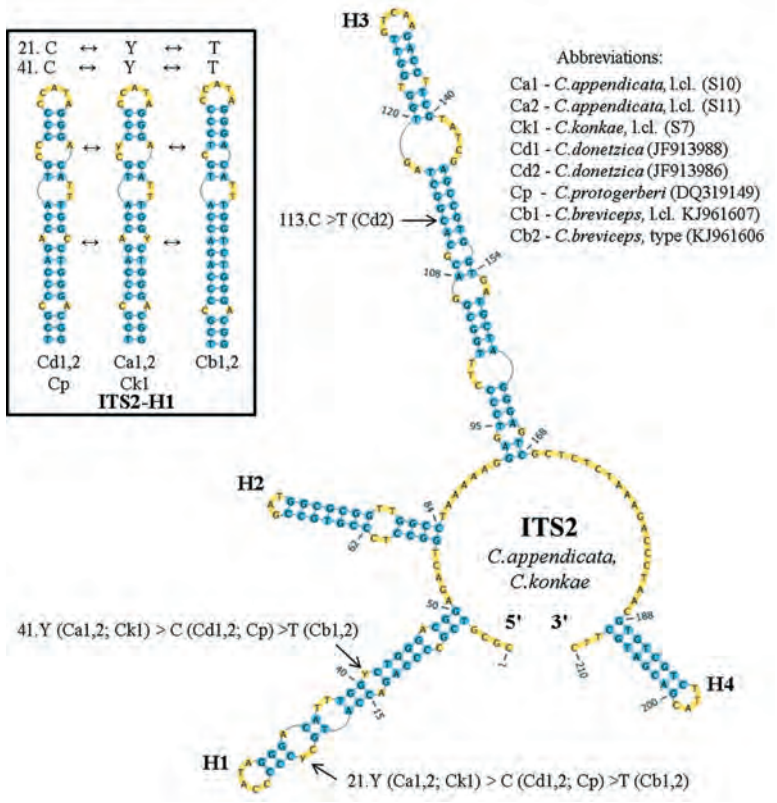

Figure 5. ITS2 secondary structure model of *C. konkae* and *C. appendicata* with variable sites that distinguish it from related species (*C. breviceps*, *C. donetzica* and *C. protogerberi*) and variants of first (ITS2-H1) helix structures of these species (highlighted frame).

Discussion

Centaurea konkae and *C. appendicata* differ from each other and from *C. donetzica* and *C. breviceps* according to morphological analysis. The main distinguishing characteristics at the morphological level are the shape and size of appendages, present keel and edge on appendages, color and size of flowers.

Centaurea konkae and *C. appendicata* ITS1 and ITS2 sequences are unique and distinguish these species from other pearl knapweeds, the corresponding sequences of which are deposited in GenBank.

Both species are very similar to each other and differ in a single site in the ITS1 second helix (site 139), where only cytosine is present in *C. appendicata* and SNP (cytosine and thymin) is found in *C. konkae*. Both species have identical ITS1 secondary structures in all possible *C. konkae* alleles of site 139. According to Coleman (2000, 2007, 2009), this fact points to a possible absence of any genetically determined incompatibility or reproductive limitation between the studied taxa.

According to ITS1 and ITS2 sequences, the *Appendicatae* 'row' of pearl knapweeds (*C. konkae* and *C. appendicata*) share highest similarity with three other species of Ukrainian pearl knapweeds and represent two different molecular micro-clades according to Hilpold's system (Hilpold et al. 2014): 'Ukraine 1' (*C. donetzica* and *C. protogerberi*) and 'Ukraine 2' (*C. breviceps*) within the Eastern ribotype Centaurea group of the Circum-Mediterranean Clade (CMC) subgenus

Centaurea. The *Appendicatae* 'row' of pearl knapweeds occupy an intermediate position between these two micro-clades. Four single nucleotide polymorphism sites in ITS1 (67 and 134) and ITS2 (sites 21 and 41) indicate the following: ITS1 and ITS2 are identical (or nearly identical) in their alternative allelic states either with micro-clade species 'Ukraine 1' (especially *C. donetzica* JF913988) or with micro-clade species 'Ukraine 2' (*C. breviceps* KJ961606, KJ961607). These data are consistent with Klokov's idea (Klokov 1935) that *C. konkae* and *C. appendicata* form an intermediate geographic group between pairs of species *C. donetzica* – *C. protogerberi*, on the one side and *C. breviceps* – *C. paczoskii*, on the other side.

The variable features in *C. konkae* and *C. appendicata* rDNA can be explained by one of the scenarios of hybrids and allopolyploids DNA evolution. Three main scenarios of rDNA evolution as a result of hybridization have been proposed up to date (Hribova et al. 2011): 1 – rDNA of both parental forms is saved in hybrids and evolves independently; 2 – rDNA of parental forms recombines to form chimeric DNA; 3 – rDNA of only one of the two original forms becomes dominant and totally replaces second parental rDNA. In case of the first scenario, the hybrids will possess SNP and the corresponding alternative alleles will be similar to those present in parental forms, provided that the total DNA is analyzed.

Thus, SNP presence in *C. konkae* and *C. appendicata* in sites that differentiate micro-clades 'Ukraine 1' and 'Ukraine 2' indicates the hybridogenic origin of *Appendicatae* 'row', where the original forms were, representatives of micro-clade 'Ukraine 1' on the one side and those of micro-clade 'Ukraine 2' on the other side. *C. donetzica* JF913988 ribotype is the most similar to the parent form of micro-clade 'Ukraine 1', and *C. breviceps* KJ961606, KJ961607 ribotype is the most similar to the parent form of micro-clade 'Ukraine 2'. The assumption confirms that *C. konkae* and *C. appendicata* are of hybridogenic origin and were formed allopatrically from interspecies hybrid populations of *C. breviceps* (micro-clade 'Ukraine 2') and *C. donetzica* (micro-clade 'Ukraine 1') at different banks of the Dnieper.

Conclusions

Centaurea konkae and *C. appendicata* are separate taxonomic units which differ on morphological level and occupy an intermediate position between *C. donetzica* and *C. breviceps* by results of morphological analysis. The unique sequences of *C. konkae* and *C. appendicata* have been confirmed by ITS1 and ITS2 secondary structure analysis. It was demonstrated that this pair of species is an intermediate group between molecular micro-clades 'Ukraine 1' (*C. donetzica* and *C. protogerberi*) and 'Ukraine 2' (*C. breviceps C. margaritacea*, *C. margarita-alba*, *C. protomargaritacea*) Eastern ribotype Centaurea group. Single nucleotide polymorphisms (SNP) are present in sites of ITS1 (67.Y, 134.R) and ITS2 (21.Y, 41.Y) of *C. konkae* and *C. appendicata,* the alternative alleles of which differentiate micro-clades 'Ukraine 1' and 'Ukraine 2'. The latter might be an indication that *C. konkae* and *C. appendicata* were formed by means of hybridization between the representatives of micro-clade 'Ukraine 1' (*C. donetzica*) and the knapweeds of micro-clade 'Ukraine 2' (*C. breviceps*).

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