Теоретичні та прикладні питання

Centaurea breviceps Iljin (Asteraceae, Magnoliophyta): neotype and its annotation according to ITS1 and ITS2 secondary structures

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Neotype for endemic species *Centaurea breviceps* Iljin (section *Pseudophalolepis*), which grows in the south of Ukraine, was chosen based on a material from a *locus classicus*. The sequences and secondary structures of the internal transcribed spacers (ITS) 1 and 2 were annotated. It has been shown that *C. breviceps* differs from all previously sequenced species of the *Acrolophus*, *Maculosae*, *Phalolepis* and *Pseudophalolepis* sections by the unique base change in the first helix of ITS2 (C > T, site 41). Within this group of sections, *C. breviceps* belongs to ribotype "A" represented by diploid and autopolyploid species but not allopolyploids. *C. breviceps* differs from the three sequenced species of the *Pseudophalolepis* section (*C. pseudoleucolepis, C. protogerberi* and *C. donetzica*) by the secondary structure of ITS1 second helix and ITS2 first helix.

Key words: Pseudophalolepis, Centaurea breviceps, neotype, endemic species, ITS1 & ITS2 secondary structures, Ukraine

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На основі матеріалу з *locus classicus* обрано неотип для ендемічного виду *Centaurea breviceps* Iljin (секція *Pseudophalolepis*), поширеного на півдні України. За первинною і вторинною структурами анотовано послідовності транскриптів ITS1 та ITS2 кластеру ядерних рРНК-кодуючих генів. Показано, що *C. breviceps* відрізняється від усіх раніше секвенованих видів із секцій *Acrolophus, Maculosae, Phalolepis* та *Pseudophalolepis* унікальною заміною (C > T, сайт 41) у першій спіралі ITS2. В межах цієї групи секцій *C. breviceps* належить до риботипу «А», який представлений дипліодними та автополіплоїдними видами і не містить аллополіплоїдів. *C. breviceps* відрізняється від трьох секвенованих видів секції *Pseudophalolepis* (*C. pseudoleucolepis, C. protogerberi* та *C. donetzica*) вторинною структурою другої спіралі ITS1 та першої спіралі ITS2.

Ключові слова: Pseudophalolepis, Centaurea breviceps, неотип, ендемічний вид, вторинна структура ITS1 та ITS2, Україна

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На основании материала из *locus classicus* выбран неотип для эндемического вида *Centaurea breviceps* Iljin (секция *Pseudophalolepis*), распространенного на юге Украины. По первичной и вторичной структурах аннотированы последовательности транскриптов ITS1 и ITS2 кластера ядерных рРНК-кодирующих генов. Показано, что *C. breviceps* отличается от всех ранее секвенированных видов из секций *Acrolophus*, *Maculosae*, *Phalolepis* та *Pseudophalolepis* уникальной заменой (C > T, сайт 41) в первой спирали ITS2. В рамках этой группы секций *C. breviceps* принадлежит к риботипу «А», который представлен диплоидными и автополиплоидными видами и не содержит аллополиплоидов. *C. breviceps* отличается от трех секвенированных видов секции *Pseudophalolepis (C. pseudoleucolepis, C. protogerberi* и *C. donetzica*) вторичной структурой второй спирали ITS1 и первой спирали ITS2.

Ключевые слова: Pseudophalolepis, Centaurea breviceps, неотип, эндемический вид, вторичная структура ITS1 и ITS2, Украина

The numerous *Centaurea* L. species, that considered to be strict regional endemics of Ukraine, were described. However, not all of these taxa are accepted as independent species. One of such examples is *Centaurea breviceps* Iljin (subgenus *Phalolepis* (Cass.) Spach, section *Pseudophalolepis* Klokov et al.), which is listed in Red Book of Ukraine, European Red List and IUCN Red List [CHERVONA…, 2009].

C. breviceps is an endemic species of the lower riches of the Dnieper. In the earlier works it was initially denoted as *C. leucolepis* DC. [LEDEBOUR, 1845], and later – as *C. margaritacea* Ten. [PACZOSKI, 1904; 1922; SHMALGAUSEN, 1897]. Moreover, J. Paczoski mentioned that *C. margaritacea* from sands of the Pivdenny Bug and *C. margaritacea* from the lower riches of the Dnieper are different and may represent two different races. But he did not make any taxonomic decision because of the lack of material. This difference also was noted by M.M. Iljin, who separated *C. breviceps* from *C. margaritacea* in 1927 (described in News of the Main Botanical Garden, 36 (1) (1927) [ILJIN, 1927]. According to M.M. Iljin *C. breviceps* differs from typical *C. margaritacea* from the lower riches of the Pivdenny Bug by smaller anthodia, double-coloured whitish-scarious scales and pink heads. The position of *C. breviceps* in *Centaurea* was determined by M.V. Klokov [KLOKOV, 1935; 1962], and later refined by D.M. Dobrochaieva [DOBROCHAIEVA, 1965]. They position *C. breviceps* into *Gerberinae* Klokov (together with *C. protogerberi* Klokov, *C. donetzica* Klokov and *C. paczoskii* Kotov) range of *Pseudophalolepis* Klokov section of *Phalolepis* (Cass.) Dobrocz. subgenus [KLOKOV, 1935; 1962]. He also extracted a special form, f. *asperula* Klokov, which differs by small setas and acute tubercles on leaves [KLOKOV, 1935]. Later a Czech botanist J. Dostal, during his work on Flora Europaea, changed the status of this taxa to subspecies *C. margaritacea* Ten. subsp. *breviceps* (Iljin) Dostál [DOSTÁL, 1976]. However in the latest lists *C. breviceps* Iljin was again described as a separate species [CHEREPANOV, 1994; CHERVONA..., 2009; MOSYAKIN, FEDORONCHUK, 1999; GLOBAL...].

Accorind to the data, provided by N.M. Shyyan, S.L. Mosyakin, M.M. Fedoronchuk [SHYYAN et al., 2010] it is necessary to choose a neotype for *C. breviceps,* while no authentic specimens of this species were preserved.

Several endemic species from south of Ukraine from *Pseudophalolepis* section (*C. protogerberi*, *C. pseudoleucolepis* Kleopow, *C. donetzica*) were studied using both morphological and molecular methods, including phylogenetic analysis of nuclear-encoding ribosomal genes and spacers ITS1 and ITS2 [GARCIA-JACAS et al., 2006; SUÁREZ-SANTIAGO et al., 2007; MRÁZ et al., 2012]. It was shown that in frame of Jaceae group (subgenus *Phalolepis* is included in this group) these species represent a so called "A" ribotype. This ribotype unites diploid and autopolyploid cornflowers and does not include any allopolyploid taxa. More detailed studies of molecular-genetic differences between Ukrainian endemic pearl cornflowers were not conducted and their taxonomic status has not been discussed.

Nowadays it is accepted that analysis of uncoding regions of ribosomal genes cluster allows to assess a level of reproductive isolation of specimens belonging to another close taxa

by analysis of differences in ITS secondary structure, in particular, by analysis of presence and quantity of compensatory and hemicompensatory base changes, presence of mutations, which cause or do not cause changes in secondary structure of separate helixes [COLEMAN, MAY, 1997; COLEMAN, 2000; MULLER et al., 2007; RUHL et al., 2009]. The level of reproductive isolation requires further taxonomic interpretation according to the molecular species concept developed by A. Coleman [COLEMAN, 2000; 2007; 2009].

Currently, the analysis of ITS secondary structure is based exclusively on ITS2. Pan-eukaryote ITS2 homologies were shown [COLEMAN, 2007] and the universal model of ITS2 secondary structure for green algae and higher plants was proposed [MAI, COLEMAN, 1997]. Specific motives and "pattern" for different groups of organisms were determined as well [COLEMAN, 2007]. Numerous genera and species of higher plants, algae, animals, and fungi were described and revised based on ITS2 secondary structure during last years.

Unlike ITS2, ITS1 secondary structure analysis is used rarely in botanical research. It is shown that ITS1 secondary structure in Chlorobionta (green algae and higher plants) corresponds to ring-like multibranch loop model with four main helices and one-four additional ones [COLEMAN, MAI, 1997; COLEMAN et al., 1998] where the third main helix contains a motive universal for higher plants: GGCRY-(4 to 7 n)-GYGYCAAGGAA [LIU, SCHARDL, 1994]. This model is validated for higher plants such as Boraginales [GOTTSCHLING et al., 2001], *Quercus* [MAYOL, ROSSELLO, 2001], Veroniceae [DIRK, CHASE, 2004] and (without publishing of secondary structure image) for some another land plants [FANTACCIONE, WOODROW, PONTECORVO, 2008; HŘIBOVÁ et al., 2011]. ITS1 seconary structure models for *Centaurea* representatives (including subgenus *Phalolepis*) have not been proposed yet.

In the present study, molecular genetic annotation of *C. breviceps* based on the specimen proposed as *C. breviceps* neotype and also two additional specimens from locus classicus and from Chornomorsky Biosphere Reserve was proposed. The level of differences between *C. breviceps* and another species of endemic pearl cornflowers of Ukraine was estimated based on ITS1 and ITS2 secondary structure.

Material and methods

In present study, we used three specimens of *C. breviceps,* collected and determined by I.I. Moysiyenko. Two specimens were collected simultaneously in *locus classicus*: Kherson region, Tsurupinsk disrict, near Tsurupinsk, reserve "Sagy", sand arena, one of two specimens was chosen as neotype (deposed in herbarium of Kherson State University as KHER 10001). The third specimen represents the population which grows on sandy arenas in Chornomorsky Biosphere Reserve (Kherson region, Hola Prystan district, Solonoozerna part of Chornomorski Biosphere Reserve, psammophytic steppe). All three specimens fully correspond to the initial morphological description of the species.

DNA isolation, amplification and sequencing. DNA was isolated by CTAB-technique [DOYLE, DOYLE, 1987] with modifications for herbarium specimens [TAREEV et al., 2011].

Amplification was provided according to [CHASSOT et al., 2001] on Techne thermocycler. Amplicons of ITS1-5.8S-ITS2 were obtained using ITS1 and ITS4 universal primers [WHITE et al., 1990]. Amplified products were commercially sequenced with identical primers (www.macrogen.com., Netherlands). Sequence editing was conducted manually through visual inspection of obtained chromatograms using BioEdit software package (www.mbio.ncsu.edu/bioedit/bioedit.html). Obtained sequences were deposited in GenBank (table 1).

Dataset for comparison was formed from ITS1 and ITS2 sequences of *C. breviceps* and the same sequences of other species of *Pseudophalolepis* section from the territory of Ukraine, available from GenBank (see: table 1).

Table 1

***Centaurea* (subgenus *Phalolepis*) species ITS1-ITS2 sequences used in analysis of *C. breviceps* taxonomical status**

Name of taxa according to GenBank and remarks	GenBank accession number
C. breviceps (neotype, KHER 10001)	KJ961606
C. breviceps (*locus classicus*, KWU 59569)	KJ961607
C. breviceps (Chornomorsky Biosphere Reserve)	KJ961608
C. protogerberi	DQ319149
C. pseudoleucolepis	DQ319150
C. pseudoleucolepis	AM114328
C. donetzica (2x)	JF913986
C. donetzica (2x)	JF913988
C. donetzica (2x)	JF913987

Annotation of ITS2 sequence was conducted by mFOLD modeling of the terminal region of 5.8S and start region of LSU secondary structure [ZUKER, 2003] (19 bp) which are complementary to each other (according to the model, proposed for dinophytes [GOTTSCHLING & PLOTNER, 2004]). The complementary terminal region of 5.8S and start region of 28S for *C. breviceps* neotype were as following:

[CGTCTGCCTGGGCGTCACG(CAT) end 5.8S] –[ITS2] – [start 28S (GAC)CGCGACCCCAGGTCAGGCG]

ITS1 sequence annotation was done by comparing the parts of *C. breviceps* sequences to the full ITS1 sequence of *C. stoebe* subsp. *micranthos* (S.G.Gmel. ex Gugler) Hayek (FJ969855), already annotated in GenBank, and assessing the degree of similarity. The sequence search was performed using a ClustalW multiple alignment algorithm in BioEdit programme.

Models of ITS1 and ITS2 secondary structures were constructed in mFOLD [ZUKER, 2003]. ITS1 secondary structure of *C .breviceps* was selected through consistent adding of helices in accordance with the model proposed for Boraginales [GOTTSCHLING et al., 2001]. According to this model, higher plants conserved motive GGCRY-(4-7 base pairs)-GYGYCAAGGAA [LIU, SCHARDL, 1994] is located in helix H3; helices H1+H2, H3 and H4 are separated by A-rich regions. ITS2 secondary structure was determined by serial H1-H4 helices' assembling. The third and the second helices were constructed initially and their folding accuracy was tested by location of the conservative motive of A. Coleman – NRTGGT [COLEMAN, 2007] in apex on 5'-side of H3 helix and by position of universal conservative pyrimidine-pyrimidine mismatch in subbasal part of H2 helix [MAI, COLEMAN, 1997]. Obtained models of ITS1 and ITS2 secondary structures were visualized by Pseudoviewer 3.0 [Byun, Kyungsook, 2006].

Results

The 700 bp long sequences, which included partial sequence of 18S rDNA (16 bp), complete sequences of ITS1, 5.8S rDNA and ITS2 (255, 166 and 210 bp respectively) and partial sequence of 28S rDNA (53 bp) were obtained for specimens of *C. breviceps* from *locus classicus*. The sequence from Chornomorsky Biosphere Reserve specimen was 632 bp in length and included complete sequences of 5.8S rDNA and ITS2, partial sequence of ITS1 (248 bp) (except 8 first nucleotides) and short fragment of 28S rDNA (8 bp). All sequences of 18S, ITS1, ITS2 and 28S were identical in overlapping regions. The only difference between the sequences was a single nucleotide polymorphism (SNP) in the site 138 of 5.8S rDNA (138.G>R) in both specimens from *locus classicus* but not from Chornomorsky Biosphere Reserve (fig. 1).

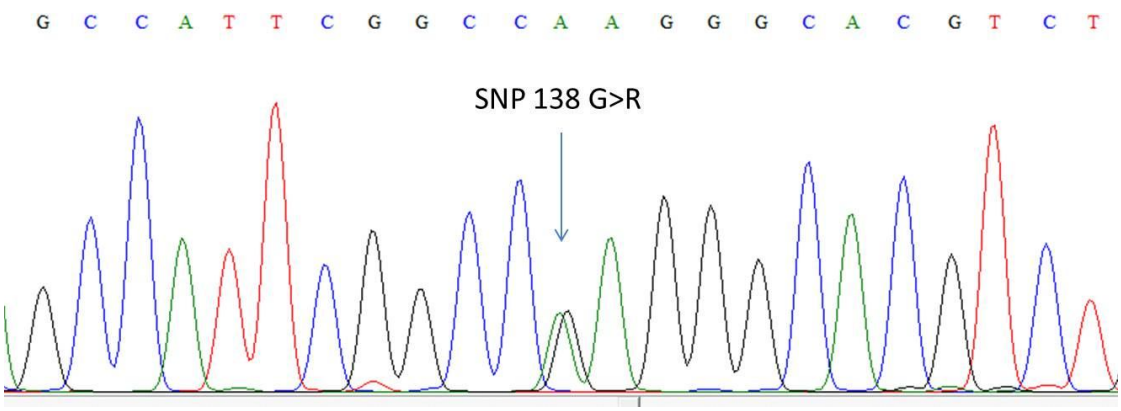

Fig. 1. Single-nucleotide polymorphism in 5.8S rDNA sequence of *C. breviceps* neotype specimen.

BLAST (http://blast.ncbi.nlm.nih.gov/) search results have shown that ITS1-5.8S-ITS2 sequnce of *C. breviceps* differed from all another cornflower sequences by at least one base change. Sequence of C. breviceps showed the 99% similarity to 22 species (86 sequences). It should be noted that all these species belong to Acrolopus-Phalolepis group (AP-group), which include *Acrolophus*, *Maculosae*, *Phalolepis* and *Pseudophalolepis* sections [GARCIA-JACAS et al., 2006]. Among them there are two species from *Acrolophus* section (*C. diffusa* Lam., *C. stoebe*), four – from *Phalolepis* section (*C. sterilis* Stev., *C. vankovii* Klokov, *C. sarandinakiae* Illar., *C. semijusta* Juz.) and three from *Pseudophalolepis* section (*C. protogerberi, C. donetzica, C. pseudoleucolepis*) which grow on the territory of Ukraine. However, specimens of *C. breviceps* differed from all another species of Acrolopus-Phalolepis group by the presence of T instead C in the site 41 of ITS2.

ITS1 secondary structure. Obtained model of *C. breviceps* ITS1 secondary structure included four main (H1-H4) and two additional (Ha and Hb) helices (Fig. 2). Both primary and secondary structures of H3 helix conformed to the reconstruction of the higher plants universal motive of Liu and Schardl [LIU, SCHARDL, 1994].

The topology of three another helices (H1, H2, H4) corresponded to generalized model of ITS1, which was proposed before for Boraginales [GOTTSCHLING et al., 2001]. The absence of additional helixes Hc and Hd was the most prominent specific feature of the secondary structure we obtained. All six sequences of three another species from *Pseudophalolepis* section (*C. protogerberi, C. pseudoleucolepis, C. donetzica*) had ITS1 secondary structures identical to *C. breviceps.* But all sequences differed from each other and from *C. breviceps* by base changes and SNP in six sites – 6, 67, 102, 125, 189, 234 (Table 2).

C. breviceps appeared to be most distant from *C. pseudoleucolepis* based on the presence of three base changes. Substitution in site 102 (G>T) of H3 helix was most significant as it caused changes in ITS1 secondary structure. Two another base changes (in sites 6.G>C and 125.C>T) in single strand regions ("loops") of Ha and H2 helices resulted in no changes in ITS1 secondary structure. *C. breviceps* was different from *C. donetzica* and *C. protogerberi* by one change in the terminal loop of H1 helix (site 67.S> T). This substitution caused changes in the secondary structure of ITS1. Moreover *C. breviceps* had minor additional differences from *C. donetzica* based on absence of single-nucleotide polymorphism in two sites of the central loop (189.C>M) and the stem of H4 helix (234.G>R).

The results of autapomorfy search, which can separate *C. breviceps* from all another 22 species of AP-group with 99 % of identity and more (according to BLAST search results) were negative.

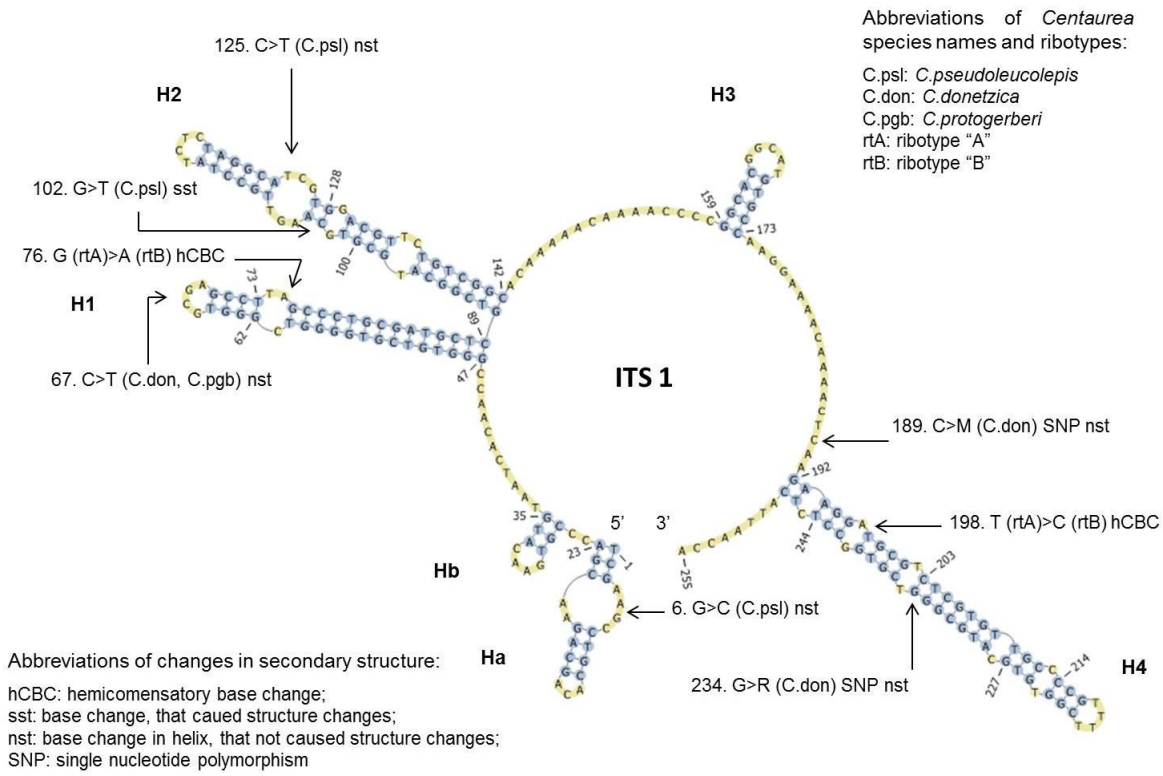

Fig. 2. ITS1 secondary structure model of *C. breviceps* neotype (variable sites that distinguish this species from other sequenced representatives of *Pseudophalolepis* section and demonstrate its affiliation to ribotype "A" are marked).

Table 2

Variable sites in ITS1 and ITS2 of studied species from *Pseudophalolepis* section

Species	Quantity of sequences	Sequence									
		ITS1						ITS2			
		№ of position in sequence						№ of position in sequence			
		6	67	102	125	189	234	21	41	107	113
C. breviceps (l.cl.)	2	G	C	G	C	C	G	T	T	G	C
C. breviceps (ChBR)	1	n/a	C	G	C	C	G	T	T	G	C
C. pseudoleucolepis	2	C	C	T	T	C	G	T	C	G	C
C. protogerberi	1	G	T	G	C	C	G	C	C	G	C
C. donetzica	3	G	T	G	C	M	R	C	C	R	Y
Type of secondary structure change		nst	nst	sst	nst	SNP	SNP	sst	sst	SNP	SNP

Abbreviations: sst – substitution, that causes changes in secondary structure; nst – substitution, that does not cause changes in secondary structure; SNP – single nucleotide polymorphism; n/a – data is not avaliable; l.cl. – specimens from *locus classicus*; ChBR – specimen from Chornomorsky Biosphere Reserve

According to literature data [MRAZ et al., 2012] there are sites with AP-group devided into two main ribotypes where ribotype "A" includes species with diploid chromosome number and ribotype "B" is represented by three- tetra- and hexaploid plants with allopolyploid origin. Our investigation of the sites showed that two of three diagnostic sites are located in ITS1. Diagnostic site "№ 77" matched site 76.G (for ribotype "A") > A (for ribotype "B") of H1 helix. Diagnostic site "№199" matched site 198 T (for ribotype "A") > C (for ribotype "B") of H4 helix. It should be noted that allele change caused hCBC creation in both sites. This fact indicates that representatives of different ribotypes do not have full

reproductive compatibility. *C. breviceps* as well as *C. pseudoleucolepis*, *C. donetzica* and *C. protogerberi* belongs to ribotype "A" in *Pseudophalolepis* section.

ITS2 secondary structure. Our attempts to use ITS2 secondary structures' models of closely related to *C. breviceps* species which are deposited in ITS2 database (http://its2.bioapps.biozentrum.uni-wuerzburg.de/) [KOETSCHAN et al., 2010] *C. pseudoleucolepis* GI 83941072 (DQ319150) and GI 121488939 (AM114328); *C. protogerberi* GI 83941071 (DQ319149) showed that the models proposed by this server did not completely correspond to the typical structure of ITS2. For instance, these models did not possess in H2-ITS2 a pyrimidine-pyrimidine mismatch described in literature [MAI, COLEMAN, 1997]. As a result, reconstruction of the whole H2 helix and the basal segments of H1 and H3 helices seemed to be doubtful. The latter was confirmed by the presence of three thermodynamically forbidden double bonds in basal segments of H1 helix as well as by the absence of such H3 helix structure variants gained with the help of mFOLD in which the basal part is identical to that proposed in ITS2 database. The improved model of ITS2 secondary structure, which lacks the abovementioned drawbacks, has been proposed. (fig. 3). Moreover, it has much lower free energy (ΔG = -90.30 kcal/mole) than models from ITS2 database (ΔG = -63.4kcal/mole).

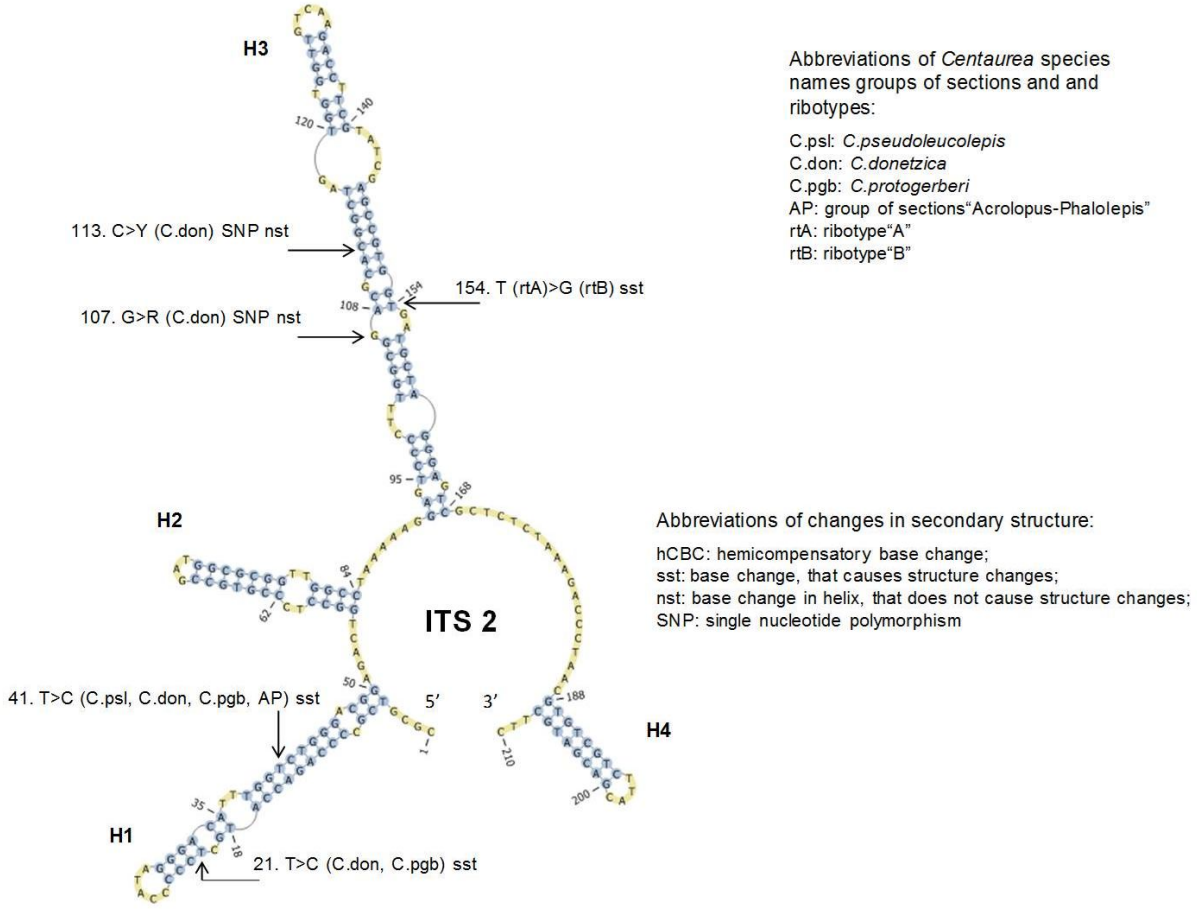

Fig. 3. ITS2 secondary structure model of *C. breviceps* neotype (variable sites that distinguish this species from other sequenced representatives of *Pseudophalolepis* section and demonstrate its affiliation to ribotype "A" are marked).

C. breviceps ITS2 secondary structure corresponded to the ring-model with four helices where H3 helix was the longest and contained A. Coleman conservative motive for higher plants, represented by GGTGGT sequence in their subapical part (121–126 sites). H2 helix was G/C rich in basal part and had T-T mismatch (Fig. 3). All six sequences of three another species of *Pseudophalolepis* section (*C. protogerberi, C. pseudoleucolepis,*

C. donetzica) had similar ITS2 secondary structure to *C. breviceps*. Main differences between sequences were in substitutions and SNP in four sites – 21, 41, 107, 113.

ITS2 of *C. breviceps* differed from another 22 species by one nucleotide substitution in site 41 (T>C). This substitution caused changes of H1 helix secondary structure. The change in this site distinguished *C. breviceps* from all another 22 species of AP-group with 99 % of identity and more according to BLAST search results. So, thymine presence in site 41 was a unique diagnostic feature for this species in this sapmpling. *C. breviceps* could also be distinguished from *C. protogerberi* and *C. donetzica* by substitution in site 21 (T>C), which also caused H1 helix secondary structure changes. In addition, *C. breviceps* differed from *C. donetzica* by SNP absence in two sites of H3 helix (107.G>R and 113.C>Y).

ITS2 contains one diagnostic site of previousely established ribotypes "A" and "B" for AP-group representatives, which was noted as site 577 in corresponding publication [MRAZ et al., 2012]. In model of *C. breviceps* ITS2 secondary structure this site corresponded to site 154 (T for ribotype "A" and G for ribotype "B"). H3 helix secondary structure of ribotype "A" (single strand loop) differs from that of ribotype "B" (double strand stem) in this site. *C. breviceps* can be placed to ribotype "A" according to thymine change in site 154 of ITS2 and to diagnostic sites of ITS1.

Discussion

Molecular data obtained from the reconstruction of ITS1 and ITS2 secondary structures of the representatives of *Pseudophalolepis* section show the absence of CBC (compensatory base change). This fact indicates that sexual interaction on gametic level is possible between all species in sampling. So, the whole sampling represents a single CBC-clade with status not lower than subfamily or genus according to A. Coleman species concept [COLEMAN, 2003; 2007].

Any differences, which could provide evidence of partial reproductive incompatibility between taxa, for instance hCBC were not observed in the frame of sampling [RUHL et al., 2009; AMATO et al., 2007]. Previously it was shown that AP-group can be devided into two ribotypes – "A" and "B" [MRAZ et al., 2012]. Ribotype "A" consists of diploid, autopolyploid and maybe – allopolyploid species, and all three species from *Pseudophalolepis* section (*C. pseudoleucolepis, C. protogerberi, C. donetzica*) are diploids. Ribotype "B" is considered only as a range of different allopolyploid species of hybrid origin. Same authors also detected three diagnostic sites, based on which these ribotypes can be distinguished. According to this *C. breviceps* belongs to ribotype "A". We have also demonstrated that nucleotide substitutions in two of these ribotype diagnostic sites are hemicompensatory. Nucleotide substitution in the third site causes changes in secondary structure.

Differences between ribotypes on the level of hCBC confirm their importance as features of incomplete reproductive compatibility with regard to the hybridization probability of dyploid (ribotype 'A') with allopoliploid (ribotype "B") species and the ability of these hybrids to give fertile offspring. This observation provides some indirect evidence the differences by hCBC could be interpreted as characters of belonging to different Z-clades according to the A. Coleman concept [COLEMAN, 2000]: limited reproductive compatibility, where sexual interaction is possible, but zygotes and offspring are either few or abortive. Since *C. breviceps* and three other previously sequenced species from *Pseudophalolepis* section belong to ribotype "A" and are not distinguished by hCBC, this group of species can be interpreted as one Z-clade.

Taxa of the same Z-clade but with differences of ITS2 secondary structure of some helices (mostly in H1 and H4) can be interpreted as representatives of different biological species [MAI, COLEMAN, 1997; COLEMAN, 2000]. Therefore presence of mutations which cause changes in ITS1 and ITS2 secondary structures (structural substitution – sst), were observed as differential characters for different biological species in one biological clade

[LIAKH O.A. et al., 2013]. We have revealed three variants of H2 helix of ITS1 and two variants of H1 heilx of ITS2 secondary structures among four studied species. Obtained results give us the possibility to delineate three independent operational taxonomical units (OTU): a) *C. breviceps*; b) *C. pseudoleucolepis*; c) *C. protogerberi* + *C. donetzica* based on transcript secondary structure (fig. 4)

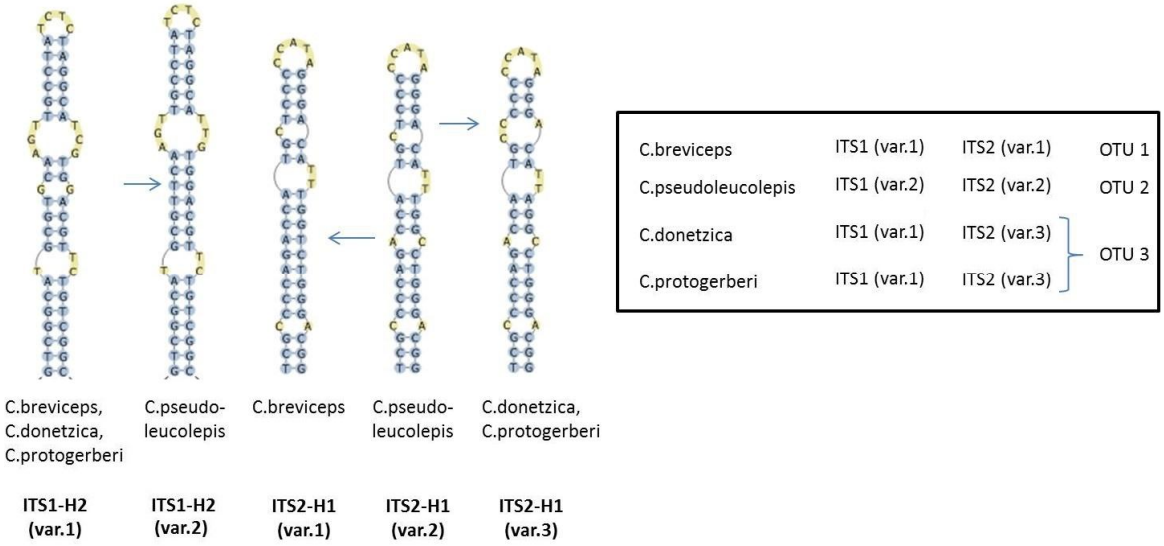

Fig. 4. Variants of ITS1 second helix and ITS2 first helix from sampling of *Pseudophalolepis* section.

Substitutions and indels in single-strand loops, especially terminal (non-structural substitution – nst), can be considered informative only on low-level taxonomy [COLEMAN, 2000]. Three detected nst's emphasize sampling delineation on three OTUs listed before. Sequences of one OTU (*C. protogerberi* + *C. donetzica*) differ by SNPs in fourth sites of ITS1 and ITS2, but SNP value as characters for delimitation of close species is still under discussion.

So, *C. breviceps* clearly differs from three another representatives of *Pseudophalolepis* section (*C. protogerberi, C. donetzica, C. pseudoleucolepis*) by ITS1 and ITS2. However, the final decision on the separate species status of this taxon depends on their similarity comparing with other taxa from this section, which have not been studied at the molecular level yet.

Conclusions

The analysis of the transcripts of ITS1 and ITS2 secondary structures revealed that *C. breviceps* within a group of related sections *Acrolophus, Maculosae, Phalolepis* and *Pseudophalolepis* (AP-group) belongs to ribotype "A", which includes diploid and autopoliploid species.

C. breviceps can be distinguished from all sequenced species from *Pseudophalolepis* section and also from all representatives of AP-group by the unique substitution (C> T) in the site 41 of the ITS2 first helix.

C. breviceps differs from studied species of *Pseudophalolepis* section by the differences in the ITS1 and ITS2 secondary structures: from *C. pseudoleucolepis* by another structure of helix 2 of ITS1, from *C. protogerberi* and *C. donetzica* by another structure of helix 1 of ITS2.

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