Genetic diversity of Bulgarian representatives of genus *Carduus* L. (Asteraceae) as revealed by variability in sequences of internal transcribed spacers region

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**ABSTRACT**

The biodiversity of genus *Carduus* in Bulgaria is grossly under-studied with modern methods, which leads to unclear status of species and hampers the assessment of their habitats and conservation status. In this study, we used the variability of internal transcribed spacers (ITS) to investigate the biodiversity of *Carduus* species collected from different floristic regions of Bulgaria. Thirty-three samples were processed. Among the studied species, *C. acanthoides* exhibited the highest ITS variability (eight single nucleotide polymorphisms were found). High ITS variability was also found in *C. crispus* and *C. hamulosis*, demonstrating that some of the local *Carduus* species possesses unique genetic diversity, which can serve as source for future divergence of new forms and subspecies. For each species, unique nucleotides suitable as molecular taxonomy markers were identified. However, *C. nutans* and *C. thoermeri* displayed completely identical ITS sequences, which is in agreement with all ITS sequences of these species collected elsewhere in the world and deposited in the NCBI database. After careful analyses of both molecular and morphological data, we propose to restore *C. nutans* and *C. thoermeri* as one species with two subspecies. Namely, *Carduus nutans* subsp. *nutans* and *Carduus nutans* subsp. *thoermeri*. We recommend diameter of capitulum, length of peduncle, width of peduncle and width of bract as diagnostic features of taxonomic significance for distinguishing the two subspecies.

**Introduction**

The genus *Carduus* L. belongs to Asteraceae family (subfamily Carduoideae, tribe Cardueae, subtribe Carduinae [1,2]. The genus is native to Eurasia (especially diverse in the Mediterranean region) and northern Africa and contains about 90 species [3]. Some *Carduus* species (C. *acanthoides* L. and C. *nutans* L.) were spread worldwide by human activities and became weedy invasive species for the Americas, South Africa, Australia and New Zealand [4,5]. The ‘Nutans’ group is a taxonomically difficult assembly of plants that include up to eight species in Europe displaying considerable variation in morphological features – hairiness, leaf-size, spine-length, width and shape of bracts, corolla-length etc. [6,7]. Bulgarian species in this complex include *Carduus thoermeri* Weimn. (syn. *C. leophyllus* Petrovic) and *Carduus nutans* (C. *nutans* ssp. *nutans*).

Unlike many other parts of the world, the current knowledge about the biodiversity of genus *Carduus* in Bulgaria is based on floristic records only. According to taxonomy studies based on morphological features in Bulgarian flora, 14 species of the genus have been described so far, all known as musk thistle. Five of these species are endemics [6,8]. They all inhabit dry grasslands and roadside locations. The endemic species are: *C. armatus* Boiss. & Heldr., *C. candicans* Waldst. & Kit. subsp. *globifer*, *C. kerneri* Simonk. subsp. *austro-orientalis*, *C. rhodopaeus* Velen. and *C. thracicus* (Velen.) Hayek.

*Carduus armatus* ( = *C. tmoleus* subsp. *armatus*) is a perennial Balkan endemic plant (South half of the Balkan Peninsula). In Bulgaria, the species is distributed in grasslands between 1500–2000 m above sea level in three floristic regions. *Carduus candicans* is a biennial to perennial Balkan endemic plant. In the Bulgarian flora, the subspecies *globifer* is presented, widespread in grasslands throughout the country, up to 1000 m altitude. *Carduus kerneri* is a perennial plant found in high mountain meadows and pastures of the South-East Carpathians and the Balkan Peninsula. The subspecies *austro-orientalis* is a Balkan endemic, found in Bulgaria and Macedonia. *Carduus kerneri* subsp. *austro-orientalis* is distributed...
from 1800 to 2800 m above sea level, in six floristic regions in Bulgaria. *Carduus rhodopaeus* (= *C. adpessus* subsp. *rhodopaeus*) is a perennial Bulgarian endemic plant, rarely distributed in the Rhodope Mountains in dry rocky places (800–2000 m altitude), with conservation status ‘Endangered’ [9]. *Carduus thracicus* is a perennial Balkan endemic plant (Bulgaria, Turkey-in-Europe), distributed in dry and waste places in Bulgaria with conservation status ‘Vulnerable’ [10]. It is very similar to *C. hamulosus*, from which it differs mainly in its smaller capitula [6].

Our understanding of *Carduus* biodiversity in the region and their local or regional distribution can be greatly enhanced by a study based on a combination of classical morphological approaches with modern molecular techniques. The present study covers the 14 Bulgarian species of the genus and combines morphological taxonomy with molecular taxonomy based on variability of the two internal transcribed spacers ITS1 and ITS2. ITS1/2 regions are among the most popular markers for studying phylogenetic inference at the generic and infra-generic levels in plants and animal species since 1994. The sequence data have provided insights into the biodiversity of closely related species, the phylogenetic history, polyploid ancestry, genome relationships, historical introgression and other evolutionary questions [11,12]. Susanna et al. [13] demonstrated the selective power of ITS1/2 molecular markers in studying the systematics of tribe Cardueae to which genus *Carduus* belongs. For this purpose, they used sequences of the nuclear ribosomal gene cluster starting from the 3’ end of the gene encoding 18S rRNA – Internal Transcribed Spacer 1 (ITS1) – the gene for 5,8S rRNA – ITS2 – and the 5’ end of the gene encoding 26S rRNA. Due to the complexity of the tribe taxonomy, they also used two chloroplastic markers (*trnL-trnF* and *matK*) to achieve more appropriate outgrouping [13].

Twenty-four accessions of the ITS1/2 region isolated from different *Carduus* species are available at the NCBI (National Center of Biotechnology Information) database, which offers excellent opportunity to confirm independently the identity of Bulgarian species by matching their ITS sequences with those annotated at the NCBI database and, on the other hand, to compare the biodiversity of the Bulgarian species with those used for molecular studies in other parts of the world.

**Materials and methods**

**Plant material**

The plant material was collected from natural habitats in Bulgaria during the vegetative seasons in 2010–2013. Thirty-three samples from all *Carduus* Bulgarian representatives collected from different floristic regions were used in this study (Table 1).

In order to minimize the harm on the *Carduus* populations, samples for DNA extraction were collected by taking a single leaf per plant from 10 randomly selected plants at each location. All leaves of one species collected from one location were stored together on dry silica gel in a refrigerator bag. The chosen method for sampling allowed us to collect samples from the same habitat more than once but at different years. In total, 33 samples for DNA extraction were collected.

**Taxonomic determination of species by morphological features**

Species identification by morphological features was done at the Department of Botany of the University of Plovdiv ‘Paisii Hilendareski’, according to Flora Europaea [6] and determination keys of Stoyanov et al. [14], Delipavlov and Cheshmedzhiev [8]. Ten morphological features with taxonomic significance were used to distinguish species: number and shape of leaf lobes; length of leaf spine; diameter of capitulum; length of peduncle; width of peduncle; width of bract; length of bract spine; length of corolla; indumentum. Fifty measurements on fresh plant material were taken for any morphological trait in each of the surveyed locations. For floristic reasons, 25 individual whole plants were collected and deposited as Voucher specimens in the Herbarium at the Agricultural University–Plovdiv, Bulgaria (Herbarium SOA).

**DNA extraction**

Upon delivery in the laboratory, the leaf samples were frozen with liquid nitrogen and ground to fine powder in pre-cooled mortar and pestle. One hundred milligrams of the plant material were used for DNA extraction by DNeasy plant mini kit (Qiagen cat. no 69,104) following the original protocol.

The absorption at 260 nm was used to determine the concentrations of the isolated DNA samples, and the ratios $A_{260}/A_{230}$ and $A_{260}/A_{280}$, to determine the presence of contaminations like proteins, polyphenolic compounds, sugars and lipids. The average amounts of isolated DNA were 250–300 ng and contaminations were present in negligible amounts.

**Primers**

We used two ITS primers from the primer Set Ribosomal primers (University of British Columbia, Nucleic Acid-
Table 1. List of taxons studied and locality of their collection.

| Species                  | Locality (latitude/longitude and altitude) | Floristic region and year of sampling | Voucher specimen (SOA-Plovdiv) |
|--------------------------|------------------------------------------|--------------------------------------|--------------------------------|
| C. acanthoides L.        | 43°08' N; 27°02' E/92 m                  | (1) Ivanski, Northeast Bulgaria, 2011 | 059717                         |
| C. acicularis Betrol.     | 41°35' N; 24°41' E/1200 m               | (2) Smolyan, Rhodope Mts (Central), 2013 | 059650                         |
| C. articulatus L.        | 42°10' N; 27°50' E/30 m                 | (3) Tzarevo, Black Sea Coast (Southern), 2010 | 059650                         |
| C. armatus Bois & Heldr. | 42°46' N; 24°58' E/1415 m               | (4) Tzarevo, Black Sea Coast (Southern), 2013 | 059781                         |
| C. candicans Waldst. & Kit. subsp. globifera (Velen.) Kazmi | 42°55' N; 26°56' E/320 m | (5) Natural park ‘Bulgarka’, Balkan Range (Central) 2012 | 059656                         |
| C. cardueus (L.) Gern.   | 42°29' N; 24°34' E/349 m                | (6) Rishki passage, Balkan Range (Eastern), 2011 | 059765                         |
| C. crispus L.            | 43°08' N; 27°02' E/92 m                 | (7) Starosel, Thracic Lowland, 2012 | 059723                         |
| C. hamulosus Ehrh.       | 43°08' N; 27°02' E/92 m                 | (8) Plovdiv, Thracic Lowland, 2012 | 060237                         |
| C. humulus L.            | 41°59' N; 24°52' E/368 m                | (9) Pamporovo, Rhodope Mts (Central), 2012 | 060242                         |
| C. kerner Simonkai subsp. austro-orientalis Franco [ = C. scardicus (Griseb.) Wettst.] | 41°50' N; 24°07' E/1550 m | (10) Kundola, Rila Mt, 2013 | 059725                         |
| C. lamarckii Boiss & Heldr. [ = C. alpestris Willd.] | 40°24' N; 23°50' E/1510 m | (11) Ivnzki, Northeast Bulgaria, 2012 | 059665                         |
| C. nutans L.             | 40°04' N; 23°50' E/1504 m               | (12) Ivnzki, Northeast Bulgaria, 2012 | 059665                         |
| C. pycnocephalus L.      | 40°04' N; 23°50' E/1504 m               | (13) Ivnzki, Northeast Bulgaria, 2012 | 059665                         |
| C. rhodopeus Velen.      | 41°40' N; 24°44' E/1431 m               | (14) Asevovgrad, Rhodope Mts (Central), 2011 | 059650                         |
| C. simum L.              | 42°08' N; 24°57' E/155 m                | (15) Panechen, Rhodope Mts (Central), 2011 | 059664                         |
| C. thracicus (Velen.) Hayek | 42°05' N; 24°28' E/217 m              | (16) Sadovo, Thracic Lowland, 2011 | –                              |
| C. thoermeri Wienm.      | 42°12' N; 25°21' E/212 m                | (17) Beglika (Forest Enterprise) Rhodope Mts (Western), 2010 | 059651                         |
| C. trachyphyllum (Petric) Stoj. & Stef. | 41°40' N; 24°44' E/1465 m | (18) Beglika, Rhodope Mts (Western), 2013 | 060238                         |
| C. trachyphyllum (Petric) Stoj. & Stef. | 41°36' N; 24°41' E/1345 m | (19) Arkutino, Black Sea Coast (Southern), 2011 | 059660                         |
| C. trachyphyllum (Petric) Stoj. & Stef. | 41°36' N; 24°41' E/1245 m | (20) Varna, Black Sea Coast (Northern), 2013 | 060236                         |
| C. trachyphyllum (Petric) Stoj. & Stef. | 41°49' N; 24°34' E/1490 m | (21) Grohtno, Rhodope Mts (Central), 2013 | –                              |
| C. trachyphyllum (Petric) Stoj. & Stef. | 43°14' N; 27°59' E/90 m | (22) Natural park ‘Bulgarka’, Balkan Range (Central), 2012 | 059780                         |
| C. trachyphyllum (Petric) Stoj. & Stef. | 43°14' N; 27°59' E/956 m | (23) Tzarevo, Black Sea Coast (Southern), 2011 | 059649                         |
| C. trachyphyllum (Petric) Stoj. & Stef. | 43°14' N; 27°59' E/956 m | (24) Tzarevo, Black Sea Coast (Southern), 2013 | 059776                         |

Note: The precise GPS coordinates of the habitat are intentionally omitted to protect Carduus populations from indiscriminate collecting of plants for medicinal and ornamental purposes.

Polymerase chain reaction (PCR)

Six independent PCR runs were performed for each sample. Approximately 150 ng DNA template was taken for each reaction and mixed with 1 μL of each primer (10 mmol/L), 25 μL PCR master mix (Fermentas, cat no K0171) and 22 μL DNase-free water (supplied with the master mix kit) in a 250-μL PCR tube. The PCR tubes were placed in a TC-512 THERMAL CYCLER (Techne, Cole-Parmer, Beacon Road, Stone, Staffordshire, ST15 OSA, UK) PCR apparatus and the PCR amplification was carried-out by using the following program: initial DNA melting at 94 °C for 5 min; next 35 cycles of 94 °C – 1 min; 55 °C – 1 min 30 s; 72 °C – 2 min 30 s and final extension at 72 °C for 6 min. The PCR products were mixed with 6.5 mL of loading dye (Fermentas #R0611), loaded onto 1% agarose gel containing 0.5 mg/mL ethidium bromide (final concentration) covered with 0.5X Tris–borate–EDTA (TBE) buffer and separated by applying 7 V/cm. The size of the products was determined by comparison with a DNA ladder (Fermentas GeneRuler ® SM0311). The PCR products were visualized by ultraviolet (UV) light.

PCR product isolation, cloning and sequencing

The PCR products were isolated from the agarose by QIAquick Gel Extraction Kit (Qiagen, cat no 28,704)
following the original protocol. The concentration of the PCR products was determined spectro-photometrically and 2–4 µL were used for A/T cloning. QIAGEN PCR Cloning Kit (cat no 231,124) was used to clone the PCR products, according to the original protocol. The ligation reactions were mixed with 250 µL freshly prepared competent bacterial (Escherichia coli-TOP 10 – Invitrogen) cells. The plasmids containing PCR products were isolated by QIAprep Spin Miniprep Kit (cat no 27,104) following the original protocol. The isolated plasmids were dissolved in 50 µL buffer (10 mmol/L Tris-Cl, pH 8.5) and sent for sequencing to GATC – Biotech AG (Cologne, Germany).

Data analysis

Online nucleotide BLAST (Basic Local Alignment Search Tool) analyses in the NCBI database were performed using the nblast algorithm of Altschul et al. [15]. The multiple alignments of obtained sequences, phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 [16]. The number of haplotypes, haplotype diversity and nucleotide diversity were calculated using DNA SP 5.10.01 software [17]. The software package Statistica v. 7.0 [18] was used for the statistical processing of morphological data.

Results and discussion

The PCR products obtained from the studied samples had an average size of 650 bp (Figure 1), whereas the size of the sequences annotated in NCBI varied between 735 and 220 bp. These differences are due to the primers combinations used. The NCBI accessions with smaller size usually contain sequences of ITS1 only. They were excluded from our further analyses. Next, the sequences of our samples were compared with those having sizes between 640 and 735 bp annotated in NCBI by other authors. The comparison demonstrated that sequences from NCBI contain longer fragments from 26S rRNA, while our sequences contained 126 bp longer fragments from 18S rRNA. Neither gene fragments encoding 18S nor 26S rRNA had any variability and, hence, no taxonomy or phylogenetic value. Therefore, they were trimmed and the size of all sequences was equalized to 524 bp. The sequences of Bulgarian representatives of genus Carduus were deposited in the NCBI database under accession numbers KT363903–KT363919.

The isolated ITS1/2 sequences were next subjected to online analyses in the NCBI database using the nblast algorithm of Altschul et al. [15]. The object of these analyses was to confirm independently the identity of wild Bulgarian Carduus species by matching their ITS1/2 sequences with those annotated at the NCBI database. Blast analyses revealed 98% similarity of Bulgarian species C. pycnocephalus and C. personata to those from the same species annotated in NCBI under accessions EF123105, AF319057 and AF319111 (C. pycnocephalus) and KM262846 (C. personata). The ITS1/2 sequences from Bulgarian C. nutans and C. thoermeri were 100% identical with accessions AY780401, EF543521, KC603920, HQ540426, AF443678 and JX867642 (C. nutans). The match between Bulgarian C. acanthoides and accession EF123106, JX867641 (C. acanthoides) was slightly lower – 96%. The lowest similarity, 92%, was observed between Bulgarian C. crispus and accession EF010530 (C. crispus from Maryland, USA), while C. crispus isolates from South Korea and China (GU188570, AY914813) showed 98 and 96% similarity, respectively.

Next, all six replicas of each Bulgarian sample were merged together to form a single consensus sequence for phylogenetic analyses. Analyses were done by MEGA6 software using the Maximum Likelihood algorithm. The joined phylogram built from ITS1/2 sequences of Bulgarian species and those annotated in NCBI (Figure 2) demonstrated satisfactory clustering by species and confirmed the accepted taxonomy scheme. The only exception was that C. thoermeri and C. nutans clustered together, which is expected due to 100% identity of their sequences. C. crispus and C. personata formed a separate branch containing a subcluster formed by C. personata samples. A relatively higher difference found in Bulgarian C. crispus is well illustrated in Figure 2, where the sequences isolated by us formed a separate subcluster but still remained in the same cluster with the C. crispus accessions annotated in the NCBI database.

Incorporation of the other eight species, which are not annotated by other authors in NCBI, did not change significantly the clustering illustrated in Figure 2, but led to formation of new clusters and subclusters specific for some of the Bulgarian Carduus representatives (Figure 3).

![Figure 1. Representative image of ITS1/2 products amplified by primers 18S Fw1 and 26S Rev1.](image-url)
All *C. pycnocephalus* sequences formed an individual cluster separated from all other species. The Balkan endemic *C. armatus* was shown to group with *C. acicularis* but they formed well distinct clusters. The other Balkan endemic, *C. thracicus*, formed a separate branch from the group of clusters accommodating *C. hamulosus* and *C. acanthoides* as distinct clusters and the joint cluster of *C. thoermeri* and *C. nutans*. The other endemic species, *C. rhodopaeus*, also formed an individual branch.

Interestingly, three species: *C. candicans*, *C. carduelis* and *C. kerneri*, displayed grouping in a joint cluster. A subtree was built only by these species (Figure 4), which demonstrated that *C. candicans* formed actually a separate subcluster, while the sequences of *C. carduelis* and *C. kerneri* are 100% identical, just like these of *C. thoermeri* and *C. nutans*.

For revision of the species taxonomy status, apart from ITS1/2 sequences as a molecular marker, profound analyses of morphological features with taxonomic importance are needed. *C. carduelis* and *C. kerneri* are rare species and we had only two specimens of the former and a single specimen of the latter, which is not sufficient for statistical analyses. Unlike them, *C. thoermeri* and *C. nutans*, are common species and we were able to record the variability of taxonomically important morphological features in 30 individual plants per species per habitat.

Leaf lobes had palmate shape in *C. nutans*, whereas triangular in *C. thoermeri*. The diameter of capitula, the

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**Figure 2.** Phylogenetic tree built by processing of ITS1/2 sequences of *Carduus* species annotated in NCBI and the isolated sequences from the same Bulgarian species.
Figure 3. Phylogenetic tree built by processing of ITS1/2 sequences of species annotated in NCBI and the isolated sequences from all 14 Bulgarian representatives from genus *Carduus*. 
length and width of peduncle, the width of bract of *C. nutans* were considerably smaller (*P > 0.001*) than those of *C. thoermeri* (Table 2).

The number of leaf lobes, the length of bract-spine and the length of corolla varied in the habitats, and in some habitats there was no statistically significant difference between *C. nutans* and *C. thoermeri* (Table 2). The statistically processed morphological data are summarized in Table 2.

| Feature | N  | L (mm) | D (cm) | P (cm) | W (mm) | B (mm) | S (mm) | C (mm) |
|---------|----|--------|--------|--------|--------|--------|--------|--------|
| Locality | X ± σ | X ± σ | X ± σ | X ± σ | X ± σ | X ± σ | X ± σ | X ± σ |
| Arkutino | 6.8 ± 0.61 | 6.8 ± 0.61 | 6.8 ± 0.61 | 6.8 ± 0.61 | 6.8 ± 0.61 | 6.8 ± 0.61 | 6.8 ± 0.61 | 6.8 ± 0.61 |
| Varna | 6.6 ± 0.58 | 6.6 ± 0.58 | 6.6 ± 0.58 | 6.6 ± 0.58 | 6.6 ± 0.58 | 6.6 ± 0.58 | 6.6 ± 0.58 | 6.6 ± 0.58 |
| Grohoto | 6.6 ± 0.65 | 6.6 ± 0.65 | 6.6 ± 0.65 | 6.6 ± 0.65 | 6.6 ± 0.65 | 6.6 ± 0.65 | 6.6 ± 0.65 | 6.6 ± 0.65 |
| Novo selo | 6.6 ± 0.58 | 6.6 ± 0.58 | 6.6 ± 0.58 | 6.6 ± 0.58 | 6.6 ± 0.58 | 6.6 ± 0.58 | 6.6 ± 0.58 | 6.6 ± 0.58 |
| Chirpan | 7.16 ± 1.23 | 7.16 ± 1.23 | 7.16 ± 1.23 | 7.16 ± 1.23 | 7.16 ± 1.23 | 7.16 ± 1.23 | 7.16 ± 1.23 | 7.16 ± 1.23 |
| Rozhen | 7.38 ± 0.50 | 7.38 ± 0.50 | 7.38 ± 0.50 | 7.38 ± 0.50 | 7.38 ± 0.50 | 7.38 ± 0.50 | 7.38 ± 0.50 | 7.38 ± 0.50 |
| Pamporovo | 7.54 ± 0.61 | 7.54 ± 0.61 | 7.54 ± 0.61 | 7.54 ± 0.61 | 7.54 ± 0.61 | 7.54 ± 0.61 | 7.54 ± 0.61 | 7.54 ± 0.61 |
| Trigrad | 6.6 ± 0.67 | 6.6 ± 0.67 | 6.6 ± 0.67 | 6.6 ± 0.67 | 6.6 ± 0.67 | 6.6 ± 0.67 | 6.6 ± 0.67 | 6.6 ± 0.67 |
| Chudnimostove | 7.42 ± 1.16 | 7.42 ± 1.16 | 7.42 ± 1.16 | 7.42 ± 1.16 | 7.42 ± 1.16 | 7.42 ± 1.16 | 7.42 ± 1.16 | 7.42 ± 1.16 |

Table 2. Comparison of morphological diagnostic features with taxonomic significance between *Carduus nutans* and *Carduus thoermeri*.

Abbreviations. N, leaf lobes number; L, leaf spine length; D, capitulum diameter; P, peduncle length; W, peduncle width; B, appendage of bract width; S, bract spine length; C, corolla length; X, mean; σ, standard deviation.
In contrast, the three studied *C. hamulosus* populations had differences in the positions and frequency of SNPs (Table 4), which indicates high genetic diversity between these populations. These findings demonstrate that this local *Carduus* species possesses unique genetic diversity, which can serve as source for future divergence of new forms and subspecies. The finding is not surprising – the Balkan Peninsula was the largest of the three refuges for plants (and other species) in Europe during the glacial period. After the melting of glaciers, that refuge became a center of biodiversity for plant species, some of which later colonized Europe [21,22]. This is the reason why the Balkan flora is rich in genetic sources and endemic plant species that have survived the glacial age.

In contrast, *C. nutans* demonstrated complete identity of ITS1/2 sequences both among sequences isolated from Bulgarian accessions and those annotated in NCBI. The fact that the ITS sequences of *C. nutans* isolated
d from Bulgaria and other places in Europe and North America are identical, confirms the weedy, invasive nature of this species [4,5]. It was obviously spread relatively recently world-wide by human activities and the time since has not been sufficient for accumulation of different mutations in distant populations even in non-functional regions like ITS.

Interestingly, the ITS1/2 sequences of *C. nutans* were identical with these of *C. thoermeri*. Earlier, *Carduus nutans* was considered as a complex group of taxonomically difficult plants [6,7]. In many earlier studies, they were considered subspecies of *Carduus nutans*—ssp. *nutans* and ssp. *leiocephalus* [14,23,24]. In contrast, based on morphological and biochemical features, Flora Europaea [6] considers them two different species: *C. nutans* and *C. thoermeri*. Exploring the karyotype of Bulgarian flowering plants, Kuzmanov et al. [25,26] reported that the *C. thoermeri* 2n karyotype is equal to 16. The same karyotype (2n = 16) occurs in *C. nutans* [27–29]. Our analyses of both molecular and morphological data, gave us sufficient evidence to propose restoration of *C. nutans* and *C. thoermeri* as one species with two subspecies,

### Table 3. List of unique nucleotides in ITS1/2 sequences in different *Carduus* species.

| Species | C. acicularis | C. armatus | C. candicans | C. cardueltis | C. crispus | C. kerneri | C. nutans | C. personata | C. pycnocephalus | C. rhodopygaeus | C. thoermeri | C. thoricus |
|---------|---------------|------------|---------------|---------------|-----------|------------|------------|-------------|-----------------|--------------|----------|----------|
|         | 199– A        | 136 – C    | 38 – G        | 116 – T       | 260 – C   | 136 – T    | 90 – T     | 462 – A      | 11 – C          | 139 – G     | 183 – A   |          |
| Position and type of nucleotide | 241 – T      |            |               | 136 – C      | 495 – C   |            | 90 – T      | 495 – C      | 346 – C         | 354 – A     |          |          |

### Table 4. List of SNPs in ITS1/2 sequences found among different Bulgarian populations of species from genus *Carduus*.

| Species | C. acanthoides | C. crispus | C. hamulosus |
|---------|---------------|------------|--------------|
| Locality | Ivanski | Smolian | Ivanski 2011 | Ivanski 2012 | Sadovo | Asenovgrad | Narechen |
| SNP position, type (rel. frequency)* | 38 | 38 | 79 | 79 | 43 | 43 | 43 |
| G(3)/T(2) | G(3)/T(2) | T(3)/C(2) | T(3)/C(2) | G(3)/T(2) |
| 43 | 43 | 136 | 136 | 149 | 149 | 149 |
| G(3)/A(3) | G(3)/A(3) | C(3)/T(3) | C(3)/T(3) | G(3)/C(3) |
| 79 | 79 | 147 | 147 | 228 | 228 | 228 |
| C(3)/T(3) | C(3)/T(3) | T(3)/C(3) | T(3)/C(3) | G(3)/C(3) |
| 82 | 82 | 149 | 149 | 149 |
| T(3)/C(3) | T(3)/C(3) | T(3)/C(3) | T(3)/C(3) | 116 |
| 116 | 116 | 198 | 198 |
| A(3)/T(3) | A(3)/T(3) | A(3)/T(3) | A(3)/T(3) | 147 |
| 147 | 147 | 149 | 149 |
| T(3)/A(3) | T(3)/A(3) | T(3)/A(3) | T(3)/A(3) | 198 |
| 198 | 198 | 198 |
| A(3)/T(3) | A(3)/T(3) | A(3)/T(3) | A(3)/T(3) | 198 |

* The numbers in brackets indicate how many of the six sequence repetitions contain a certain nucleotide.
namely, Carduus nutans subsp. nutans and Carduus nutans subsp. thoermeri. As diagnostic features of taxonomic significance for distinguishing the two subspecies, we recommend the diameter of capitulum, the length of peduncle, the width of peduncle and the width of bract.

Conclusions

For the first time, ITS1/2 sequences of Balkan endemic species were deposited at NCBI. We believe these data can support work of other researchers studying the origin, colonization ways and evolution of Carduus species. The variation of the morphological features used in the taxonomy of the genus supports molecular genetic data that C. nutans and C. thoermeri are not two separate species. Therefore, we propose the taxonomic status of the two species to be revised accepting again that there is one species, C. nutans, with two subspecies: C. nutans L. subsp. nutans and C. nutans L. subsp. thoermeri. We recommend, as morphological diagnostic features of taxonomic significance for distinguishing the two subspecies, to be used the capitulum diameter, peduncle length, peduncle width and bract width. More specimens, however, are needed before a similar taxonomic revision could be eventually proposed about the other two Carduus species, C. carduelis and C. kerneri subsp. austro-orientalis.

Disclosure statement

The authors have declared that no competing interests exist.

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