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LEAF WIDTH, nrDNA AND cpDNA ITS SEQUENCE VARIATION WITHIN CENTRAL EUROPEAN *BULBOCODIUM VERNUM* AND *B. VERSICOLOR* (COLCHICACEAE) POPULATIONS: ARE THERE REALLY TWO TAXA?

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The taxonomy of the genus *Bulbocodium,* in which two European species, a smaller eastern *(B. versicolor)* and a more robust western *(B. vernum)* are included, has been controversial since the description of the eastern species in 1821. Nuclear encoded ribosomal DNA ITS1 and the entire chloroplast DNA ITS were sequenced from several European populations, from France to the Ukraine, and the leaf width of mature living individuals was measured and analysed by ANOVA and Tukey-test. Although the studied DNA regions proved to be invariable, leaf width shows extreme variability. We found no correlation between the leaf size of the individuals and the geographical position of the populations, and in addition, the sequenced DNA regions showed total uniformity. Thus, our results do not support the division of the genus *Bulbocodium* into two taxa, at least in the sampled area. The formerly described size variants can be treated taxonomically at the *forma* level.

Keywords: Bulbocodium taxonomy – cpITS sequencing – leaf width statistics – nrITS sequencing – Tukey-test

INTRODUCTION

The distribution range of the genus *Bulbocodium* is extended from the Pyrenees in Spain to the river Volga and the Caucasus. However, the relatively small populations are scattered through this huge area, and are strictly confined to local geographic regions (valleys, basins, etc.) [6, 11, 26], therefore, the area is disperse.

Colchicum-like plants were discovered in the Pyrenees in the late 1500s by Clusius [4, 5], and they were described as *Bulbocodium vernum* by Linnaeus [10]. In the early 1800s, further specimens were reported from the Caucasus by Marschall-

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Bieberstein [12]. Presumably, cultivated material of Russian origin was described as *Colchicum versicolor* [9], and then recombined as *Bulbocodium versicolor* by Sprengel [22]. Several synonyms of this taxon were described later (e.g. *Bulbocodium ruthenicum* Bunge).

The extreme variability of the taxa, and the necessity of thorough examination of populations were emphasized by Ascherson & Graebner [1], who claimed first that the taxonomy of *B. versicolor* is very confusing ("sehr kritische Pflanze").

Serious difficulties in the distinction of the two species were reported in the early 1900s based on herbarium materials and replantation experiments [21, 24]. Several authors claimed later that the distinctive characters were not stable [13–18]. One of these characters was the base of the perianth segments (auricles), frequently measured by several authors [1, 19, 21, 30], but turned to be useless in taxonomic sense.

In spite of these uncertainties, Valentine in Flora Europaea [26] reports two distinct taxa, *B. vernum* and *B. versicolor* (Fig. 1), based on the size of the corm and the flowers, the width of the leaves, and the differences at the base of perianth segments ("acute teeth, obtuse auricles"), with none of these distinctive characters ever confirmed by the authors mentioned above. Moreover, Flora Europaea claims that the two taxa have different distribution, roughly separated by the border between Austria and Hungary (excluding the localities of *B. versicolor* in Central Italy) [26].

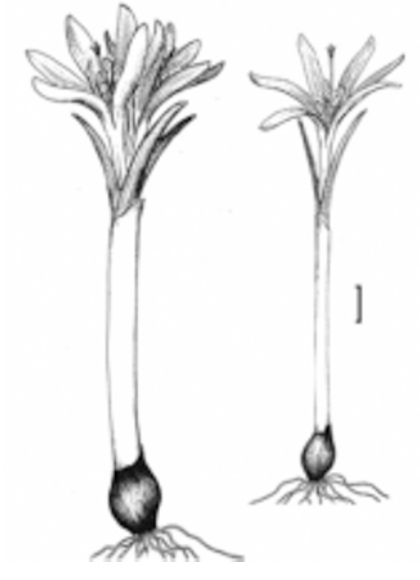

Fig. 1. Morphological extremes within the genus *Bulbocodium: B. vernum* (left) and *B. versicolor* (right). Drawings by J. Kóra using individuals collected at Tura (Hungary) and Cluj-Napoca (Romania), respectively. Scale bar: 1 cm

A karyological study by Wetschnig [28] found three studied populations (two of *B. vernum* and one of *B. versicolor*) to be distinctive by the morphology of satellite bearing chromosomes, however commenting that "in this respect there are also differences between the two populations of *B. vernum*". Vinnerstein & Reeves [27] reconstructed the phylogeny of Colchicaceae including *B. vernum* based on three plastid regions, and suggested that the separation of *Bulbocodium* from *Colchicum* is appropriate, but they could not deal with the taxonomic problem within *Bulbocodium*.

It is obvious that herbarium material is not adequate for taxonomic examinations in the genus, because the methods of preparation and the dates of collection vary extremely between herbarium specimens. Moreover, populations are represented only by few individuals, hence we get only a partial picture on the variability of the populations. In addition, our preliminary studies revealed that examination of floral parts, such as length, width and the appendices of perianth segments leads to unreliable results, mostly attributable to the rapid growth of these parts during flowering time. Taken conservation concerns into account, observation of below ground parts is impossible, so our study only uses leaf width from the morphological characters. Leaf width of a given individual does not change during the ripening time of the capsule, thus providing a relatively long time to complete the observations.

Nuclear ribosomal ITS sequencing is an effective tool in resolving angiosperm phylogeny [3]. Surprisingly, in spite of the increasing number of papers on ribosomal DNA sequences, there are no records available on this region from Colchicaceae by this time, and there is only one record on the whole rDNA (18S from *Colchicum autumnale* [20]) in GenBank. In this respect, we looked forward to obtain data not only on *Bulbocodium* populations but also on Colchicaceae as a whole.

MATERIALS AND METHODS

Plant material

Leaf width of three populations considered traditionally as *B. vernum* and nine populations of *B. versicolor* were measured from eleven different locations (pop. 1–12, Table 1, Fig. 2). The two species were distinguished based on their geographical ranges as suggested by Flora Europaea [26].

For DNA sequence analysis, one additional sample was collected (pop. 13). All samples were fresh leaves stored in absolute alcohol, completely dried before extraction.

One sample of *Colchicum autumnale* L. was used for sequence comparison, i.e. to demonstrate the sequential difference between the closely related *Bulbocodium* and *Colchicum* genera.

Table 1
Codes, locality information, vouchers and GenBank accession numbers for the studied populations of *Bulbocodium vernum, B. versicolor* and *Colchicum autumnale*

Nr.	Taxon	Sites	Voucher	GenBank accession no. nrITS, cpITS
1.	*B. vernum*	Séranon, France	*Gulyás* 622a-4 (DE)	AJ876738, AM422372
2.	*B. vernum*	Eischoll, Switzerland	*Gulyás* 622a-5 (DE)	AJ876739, AM422373
3.	*B. vernum*	Annenheim, Austria	*Gulyás* 622a-6 (DE)	AJ876740, AM422374
4.	*B. versicolor*	Tura, Hungary	*Gulyás* 622-5 (DE)	AJ876741, AM422375
5.	*B. versicolor*	Debrecen-Bánk, Hungary	*Gulyás* 622-6 (DE)	AJ876742, AM422376
6.	*B. versicolor*	Újléta, Hungary	*Gulyás* 622-7 (DE)	AJ876743, AM422377
7.	*B. versicolor*	Hosszúpályi, Hungary	*Gulyás* 622-8 (DE)	AJ876744, AM422378
8.	*B. versicolor*	Ásotthalom1, Hungary	*Gulyás* 622-9 (DE)	AJ876745, AM422379
9.	*B. versicolor*	Ásotthalom2, Hungary	*Gulyás* 622-10 (DE)	AJ876746, AM422380
10.	*B. versicolor*	Kelebia, Hungary	*Gulyás* 622-11 (DE)	AJ876747, AM422381
11.	*B. versicolor*	Subotica, Serbia	*Gulyás* 622-12 (DE)	AJ876748, AM422382
12.	*B. versicolor*	Cluj-Napoca, Romania	*Gulyás* 622-13 (DE)	AJ876749, AM422383
13.	*B. versicolor*	Shandra, Ukraine	*Melnik* 622-14 (DE)	AJ876750, AM422384
14.	*C. autumnale*	Bedő, Hungary	*Gulyás* 626-23 (DE)	AJ876751, AM422385

Fig. 2. Sampling sites of *Bulbocodium vernum* (▲), *B. versicolor* (●) and *Colchicum autumnale* (■) mentioned in text and tables. Sites "8" and "9" are very close and are not distinguished

Measuring leaf width and statistical analysis

Leaf width was measured roughly two-four weeks before full ripening of the capsule, in 2003 and 2004. Width of the largest leaf of 45–203 individuals per population was measured. The variance of leaf width among and within populations was analysed with single-factor ANOVA whereas multiple comparisons among populations were performed using the Tukey test for unequal sample sizes [31].

DNA extraction and PCR

The DNA extraction procedure was carried out according to the CTAB method of Doyle and Doyle [7] with some modifications. The incubation was at 65 °C for 60 minutes, the samples were centrifuged at 20,000 g for 10 min, then the supernatant was extracted with an equal volume of chloroform and centrifuged for 15 min at 20,000 g. The extraction procedure was repeated twice. DNA was precipitated with two volumes of ethanol and stored at –20 °C or below for 1 h. DNA was pelleted by centrifugation at 20,000 g for 30 min. The pellet was washed twice with 70% ethanol, dried and redissolved in 70 ml 0.1M Tris (pH 7.5).

For amplifying ITS, the PCR reaction mixture contained 0.1 volume 10× PCR buffer (Zenon), 200 μM each of dNTPs (Fermentas), 2 mM $MgCl_2$, 0.2 μM of each primer, 1.25 U Taq DNA polymerase (Zenon) and 5 ng/μl genomic DNA extract. The amplifications were performed on a Perkin Elmer PCR System 2400, programmed for a denaturation step at 94 °C for 4.30 min, followed by 33 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 51 °C and extension for 30 s at 72 °C, the extension time being increased by one second in every cycle; the thermal cycling was ended by a final extension for 7 min at 72 °C.

Primers and sequencing

The nuclear encoded ribosomal ITS1 (nrITS1) region was amplified by the universal primers ITS1 and ITS2 [29], however, multiple bands were sometimes obtained. In these cases, the fungus specific primer ITS1F [29] was used to detect the presence of fungi in the plant materials and the newly devised plant-specific ITS1A (5'-GACGTCGCGAGAAGTCCA) and ITS1P (5'-CCGTACCATTTAGAGGAAG-GAG) primers were applied in semi-nested PCR to amplify the plant nrITS1 specifically.

The chloroplast ITS (cpITS) region (cpITS2 – 4.5S gene – cpITS3) was amplified by the newly designed primers BKS1 (5'-TAAGCCCACCCCAAGATGAGTG) and BKS2 (5'-AGCTATTTTGCCGCAGGACC) which amplifies the spacer regions between the chloroplast encoded 23S, 4.5S and 5S ribosomal genes included the 4,5S gene itself. The primers were designed using the sequences of *Triticum aestivum* (AB042240), *Oryza sativa* (X15901), *Arabidopsis thaliana* (AP000423), *Zea mays*

(X86563), *Alnus incana* (M75719) and *Pinus thunbergii* (D17510), all retrieved from GenBank, and using standard methods and on-line programs (Oligonucleotide Properties Calculator – http://www.basic.northwestern.edu/biotools/oligocalc.html; GeneWalker – http://www.cybergene.se/primerdesign/genewalker/genewalker11.html).

For direct sequencing, the PCR products were visualised on 1% agarose gel by ethidium-bromide staining (Fig. 3, Fig. 4), and bands were purified using Montage centrifugal unit (Millipore). ABI Prism BigDye Terminator Cycle Sequencing Ready

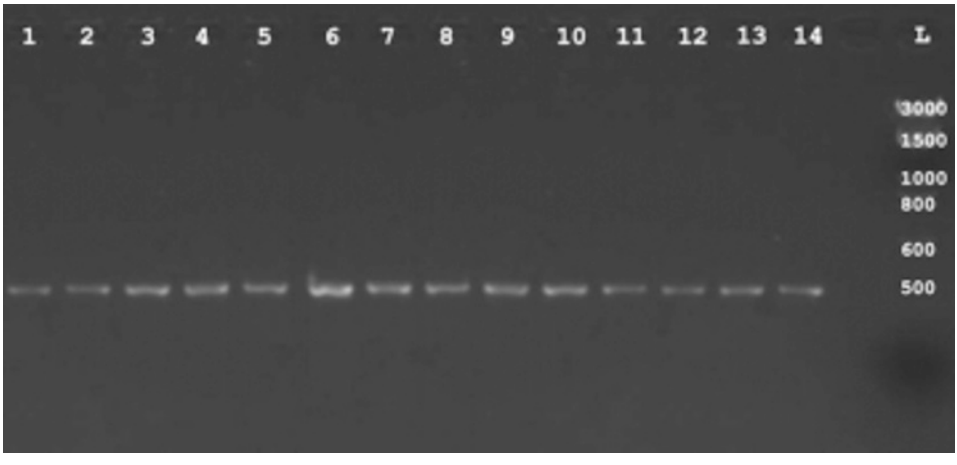

Fig. 3. cpITS PCR products of the studied populations on 1% agarose gel. The digits correspond to Table 1, the letter "L" refers to Fermentas GeneRuler 100 bp Ladders Plus, and the size of the molecular weight is also indicated

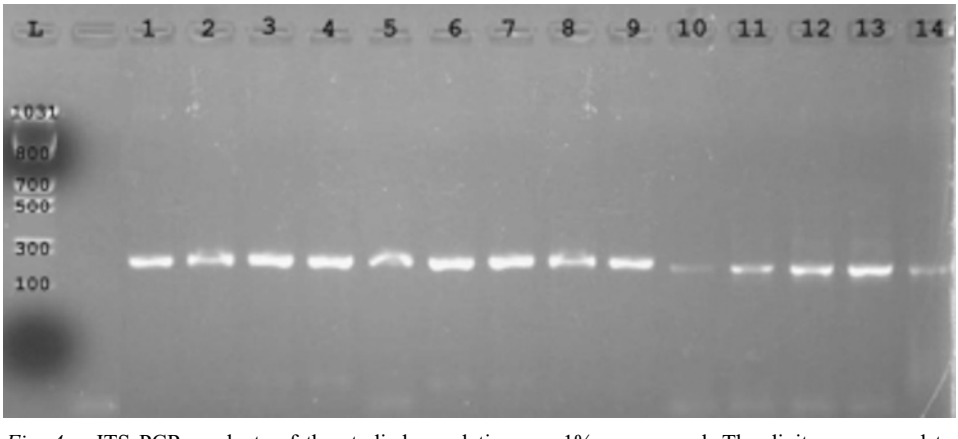

Fig. 4. nrITS PCR products of the studied populations on 1% agarose gel. The digits correspond to Table 1, the letter "L" refers to Fermentas GeneRuler 100 bp Ladders, and the size of the molecular weight is also indicated

Reaction Kit v.3.1 (Applied Biosystems) was used for cycle sequencing and electrophoresis was carried out on an ABI Prism 310 Genetic Analyser (Applied Biosystems) according to the manufacturer's instructions. Sequences were aligned by the ClustalW [25] program with default settings.

RESULTS

Leaf width

Considerable within-population variation of leaf width was detected. The variance ratio of leaf width proved highly significant ($F_{11;1469} = 90.61$, $p < 0.001$) among groups but without a clear geographic pattern. Based on the results of the pairwise Tukey tests ($p < 0.05$), the populations can be grouped as shown in Table 2. Here again, no clear geographic pattern was found (Fig. 5).

We found leaf width more variable than reported earlier: extremes were 5 mm and 45 mm. The widest leaf was found in Central Hungary (Tura, pop. 4). Though most Hungarian populations were narrow-leaved, the variation among studied Hungarian populations depicted 83% of the total variation of leaf width (Tura vs. Ásotthalom1). The difference of this characteristic between two, geographically very close populations (Ásotthalom1 and Ásotthalom2, only 8.5 km apart) was alone 60% of the total variation. However, leaf width of Ásotthalom2 did not differ significally from that of Eischoll, Switzerland, more than 1000 km (!) apart.

The geographical pattern of the leaf-width groups inferred from between-population differences based on Tukey-tests shows no congruence with the areas of the presumed taxa (Fig. 3), although leaf-width is considered as a distinguishing feature between the two presumed *Bulbocodium* species according to Valentine [26].

Table 2

Leaf width of the studied *Bulbocodium* populations (mean ± s.e.; mm).
Letters (a–d) refer to significant ($p < 0.05$) between-population differences based on Tukey-tests

Population code	Taxon	Site	Mean ± s.e.	n
3.	*B. vernum*	Annenheim, Austria	20.52 ± 6.53^{a}	46
4.	*B. versicolor*	Tura, Hungary	18.79 ± 7.51^{a}	198
9.	*B. versicolor*	Ásotthalom2, Hungary	16.19 ± 7.24^{b}	100
2.	*B. vernum*	Eischoll, Switzerland	15.49 ± 5.13^{bc}	100
1.	*B. vernum*	Séranon, France	13.74 ± 3.84^{c}	76
6.	*B. versicolor*	Újléta, Hungary	11.80 ± 2.56^{c}	100
11.	*B. versicolor*	Subotica, Serbia	11.60 ± 3.69^{cd}	102
7.	*B. versicolor*	Hosszúpályi, Hungary	9.71 ± 2.27^{d}	161
10.	*B. versicolor*	Kelebia, Hungary	9.52 ± 3.03^{d}	101
5.	*B. versicolor*	Debrecen-Bánk, Hungary	9.36 ± 3.46^{d}	196
8.	*B. versicolor*	Ásotthalom1, Hungary	9.33 ± 2.76^{d}	201
12.	*B. versicolor*	Cluj Napoca, Romania	9.08 ± 3.10^{d}	100

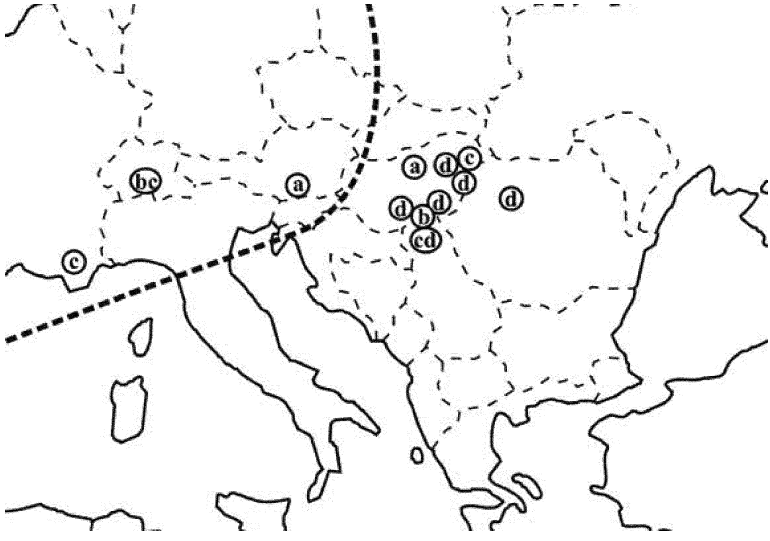

Fig. 5. Geographical pattern of leaf width groups in the studied *Bulbocodium vernum* and *B. versicolor* populations based on Tukey-tests. The abbreviations are as in Table 2, letters are referred to the same population as positioned at the same place in Figure 2. Dashed-line refers to the presumed geographic limit between the two taxa (after Valentine [26])

DNA sequence analysis

Direct sequencing of the cpITS region resulted in identical 428 bp sequences in *B. vernum*, *B. versicolor* and *Colchicum autumnale* (Fig. 6).

Similarly to the latter, sequencing of nrITS1 resulted in identical 214 bp sequences in both *Bulbocodium* species. The sequences were abruptly cut off at the 214th base, thus, only partial sequences were yielded. This may responsible for the lack of nrITS data of Colchicaceae species in GenBank. The alignment of the partial nrITS1 sequences revealed only 1.4% (3 bp) differences for *C. autumnale* (Fig. 7).

DISCUSSION

Considering their presumed habitat preference, it can be well explained why the two forms are still distinguished from each other: *B. vernum* is thought to be an alpine plant [8] living in the Pyrenees and the Alps between 1000–2500 m, while *B. versicolor* is a forest-steppe species [2], restricted to relatively low altitudes. Though *B. versicolor* can be found in the Caucasus and in the Apennines as well, their dissimilar habitat preference could easily maintain most authors' opinion on the existence of two species.

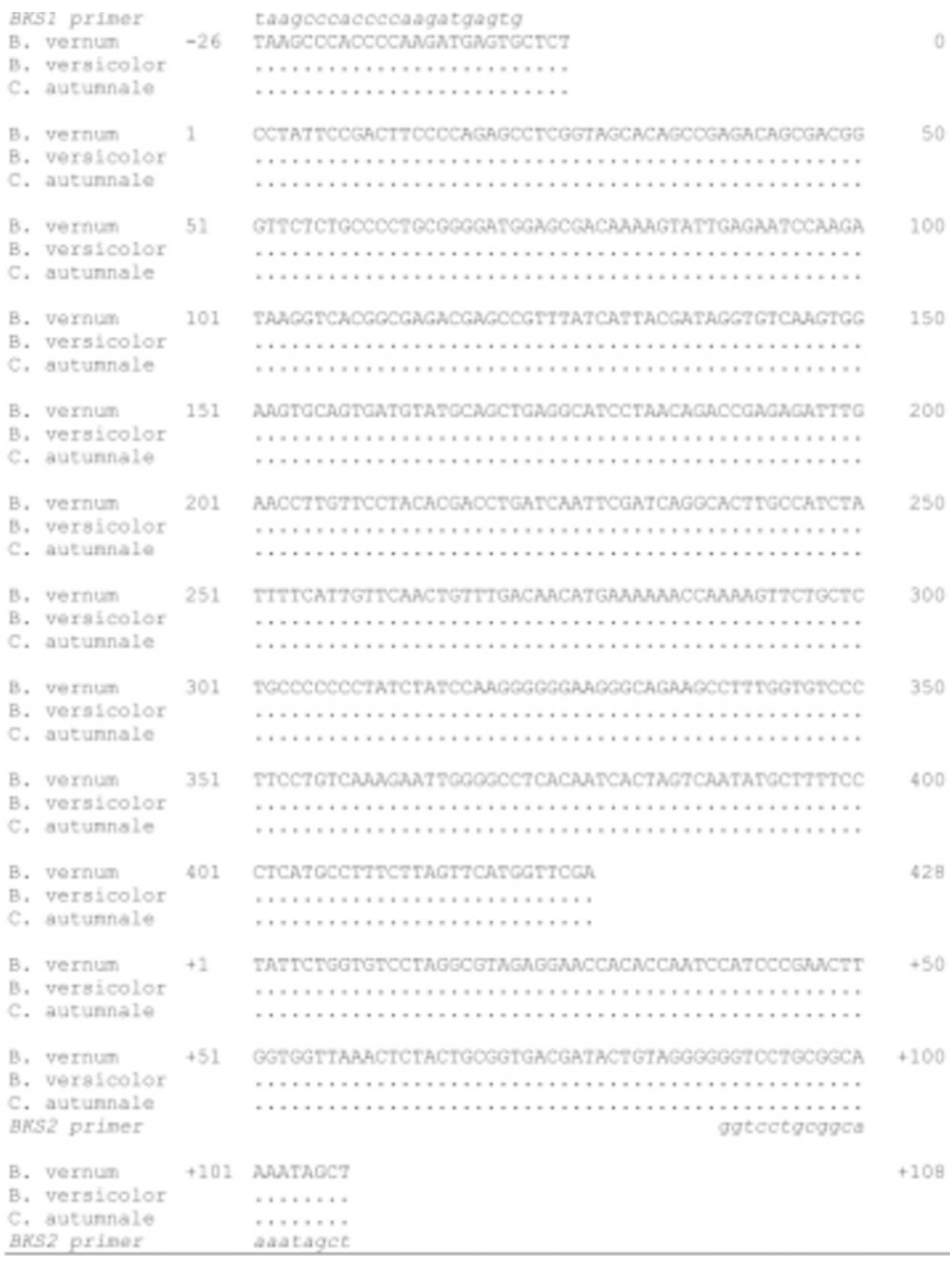

Fig. 6. Partial 23S gene, entire cpITS and partial 5S gene sequences of the studied taxa with the primer binding sites. Dots indicate identical nucleotides

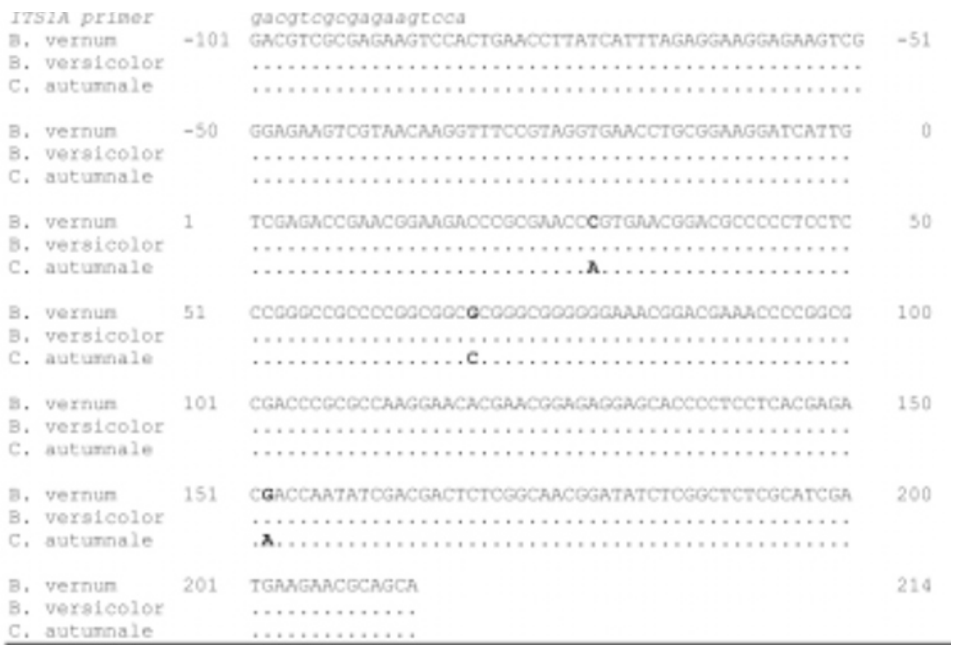

Fig. 7. Partial 18S gene and partial nrITS1 sequences of the studied taxa with the primer binding places. Dots indicate identical nucleotides

We consider Ker-Gawler's description of *Colchicum versicolor* as the origin of the taxonomic problem. The description [9] was based on only one individual, and the bulb was sent him from Moscow, consequently, he could not know adequately the species. Moreover, there was no differential character (viz. from *Colchicum bulbocodium*, as he knew the other species) in the diagnosis. Interestingly, he claimed that it was "under the appellation of Amaryllis colchiciflora" and "probably unknown to any botanist", which was rather surprising after Marschall-Bieberstein's work [12]. The most peculiar thing was that the individuals were flowering during autumn (!) as claimed by Ker-Gawler.

As a whole, we can say that Ker-Gawler's distinction of *C. versicolor* was unsubstantiated, and this fact was only recognized by Stefanoff [23]. Surprisingly, discussion of differences between the two taxa was started more than 80 years after the description [1], even without emphasizing these differences by any other author before.

An additional cause of the taxonomical problem is the lack of studies comprising the entire distribution range, and that most researchers examined only a few individuals (moreover, mainly herbarium material) and only from a small fraction of the area of the species.

Our results show that the sequenced nrITS1 and cpITS regions are conservative in *Colchicum* and *Bulbocodium* genera so that these regions are not suitable to reveal taxonomic relationships.

The high variation of leaf width is in accordance with the observation of Ascherson & Graebner [1] and Tamássy [24], who reported small individuals in *B. vernum* and robust individuals in *B. versicolor* populations. The lack of congruence between the morphological character and the geographical pattern of the presumed taxa and the high concordance of the studied molecular markers in our results do not support the separation of genus *Bulbocodium* into two species or subspecies, at least in the study area.

In our view, the high levels of variability in size do not correspond with the biogeography of *Bulbocodium* populations. This may describe a species the area of which, perhaps as a member of a forest-steppe or steppe zone once drawn across Europe, is completely fragmented by this time. Notwithstanding, there is stark contrast between habitats of the taxa (alpine meadows vs. steppe grasslands) nowadays, which would lead to total genetic separation in the future. However, the incongruence between the distinguishing morphological feature (size of the leaf) and the presumed taxa is conspicuous, and, coupled with the invariability of the ITS regions, may refer to ecotypes of the same species, namely *Bulbocodium vernum* L. If one insists on the taxonomical classification of the ecotypes, since size of the plant is only influenced by its fitness, and this character is not heritable [24, and own observations], they can be ranked at the form (*forma*) level, rather than at the species (i.e. as *B. vernum* L. and *B. versicolor* (Ker-Gawl.) Spr.) or at the subspecies (*Bulbocodium vernum* L. subsp. *vernum*; *B. vernum* L. subsp. *versicolor* (Ker-Gawl.) Richter) level.

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