

CHROMOSOME COMPLEX OF THE RELICT DIPLOID SPECIES *HIERACIUM BRACTEOLATUM*

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In the predominantly polyploid and apomictic genus *Hieracium* (Asteraceae) sexual diploids are extremely rare and their distribution is limited mainly to the refugial areas of southern Europe. Here we characterized for the first time the chromosome complex of the relict species *Hieracium bracteolatum* from a diploid population on the Greek Island of Evia. The cytogenetic analysis based on classical chromosome staining, C-banding/DAPI method and fluorescence *in situ* hybridization with rDNA probes (rDNA-FISH) showed no major differences in the karyotype structure between this relict species and other diploids within the genus, especially in terms of chromosome morphology and rDNA location. Our study is part of the still very scarce research on the karyotype organization in sexual *Hieracium* taxa.

Keywords: C-banding/DAPI, diploids, FISH, *Hieracium bracteolatum*, karyotype, rDNA.

INTRODUCTION

In Europe the genus *Hieracium* L. s.str. is dominated by polyploids, mostly tri- and tetraploid, hybridogenous taxa reproducing apomictically (diplospory of the *Antennaria* type), which is a consequence of the multiplication of the $x = 9$ genome (Asker and Jerling, 1992; Chrtek et al., 2004, 2007, 2020; Fehrer et al., 2009; Mráz and Zdvořák, 2019). Sexual diploids are very rare and occur mainly in refugial areas of southern Europe, especially on the Balkan and Iberian Peninsulas, where *Hieracium* flora is largely endemic (Merxmüller, 1975). According to Schuhwerk and Lippert (1998), the share of diploid *Hieracium* species on the Balkan Peninsula reaches, however, only up to 5% of hawkweeds occurring there, whereas on

the Iberian Peninsula it is nearly 24%. Merxmüller (1975) considered this difference a result of the poor karyological knowledge of the Balkan hawkweeds. Indeed, the intensive studies focused on determination of the chromosome number, carried out in the last two decades in the Balkans, resulted in discoveries of other new diploid *Hieracium* species or diploid cytotypes of previously known polyploids (Vladimirov, 2003; Vladimirov and Szelağ, 2006; Szelağ, 2010; Szelağ et al., 2007; Szelağ and Ilnicki, 2011).

One of the most interesting *Hieracium* species on the Balkan Peninsula is *H. bracteolatum* s.lat. recently found in diploid populations in Greece (Musiał and Szelağ, 2019; Vladimirov unpubl.). Previously triploid populations had been found (Chrtek, et al. 2009) and in 2020 Musiał et al.

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determined a somatic chromosome number $2n = 4x = 36$ in specimens of *Hieracium bracteolatum* subsp. *koracis* (Giona Mts., Greece).

Here we present results of the cytogenetic analysis of diploid *H. bracteolatum* ($2n = 2x = 18$).

This morphologically outstanding Circum-Aegean relict species had not been studied in terms of karyotype organization before. In our study we used conventional chromosome staining as well as fluorescent methods such as fluorescence *in situ* hybridization (FISH) with rDNA probes and C-banding/DAPI differential staining.

MATERIAL AND METHODS

The seeds of *H. bracteolatum* were collected from plants within a diploid population on the Greek Island of Evia (Mt. Xeroboúni massive, 920 m a.s.l.). Plants derived from the seeds were grown in pots, in a vegetation room at a temperature of 19°C, under horticulture grow lights (Phytolite HPS Bloom Spectrum 400W) with a photoperiod of 12 hours (Fig. 1).

The radicles were collected, pretreated with saturated solution of α -bromonaphthalene for 24 h at room temperature and fixed in a mixture of glacial acetic acid and absolute ethanol (1:3, v/v), then stored at 4°C. Before squashing in 45% acetic acid, fixed root tips were hydrolyzed for 10 min in 1 M HCL at 60°C (in the case of conventional chromosome staining with 0.1% aqueous solution of toluidine blue). For differential chromosome staining with C-banding/DAPI method and FISH, root tips were macerated enzymatically (4% pectinase + 2% cellulase in 0.01 M citric buffer, pH 4.6-4.9) at 37°C for 15 min.

C-banding/DAPI on squashed *H. bracteolatum* preparations was conducted according to Grabowska-Joachimciak et al. (2011). Probe labelling and FISH followed the procedure described by Wolny and Hasterok (2009) with minor modifications mentioned below. For visualization of 5S rDNA loci the probe was synthesized by PCR amplification of the pTa794 clone containing a 410 bp fragment of 5S rDNA unit isolated from wheat (Gerlach and Dyer, 1980) and labelled with digoxigenin-11-dUTP (Roche). The digoxigenated probe was immunodetected according to standard protocols by antidigoxigenin antibodies conjugated with fluorescein isothiocyanate (FITC; Roche). The probe for 45S rDNA loci was based on a 2.3 kb *Cla*I subclone representing the fragment of a 25S rDNA gene



Fig. 1. Pot-grown diploid ($2n = 18$) specimen of *Hieracium bracteolatum*; visible magnified fragment of the inflorescence stem.

region of *Arabidopsis thaliana* (Unfried and Gruendler, 1990), generated by nick translation and labelled with tetramethyl-rhodamine-5-dUTP (Roche). The slides with hybridization mixtures were denaturated at 70°C for 5 min.

Chromosome observations were made under a Nikon Eclipse E800 microscope and the images were captured and processed with a Nikon DS-2MBWc camera and the NIS Elements software. Fifteen well-spread conventionally stained metaphase plates were selected for the karyotype analysis and chromosome measurements were performed using the KaryoType program (Altinordu et al., 2016). The chromosome types were classified according to Levan et al. (1964), based on the averaged results of chromosome arm length measurements and the arm ratio ($r = q/p$).

RESULTS AND DISCUSSION

Despite new data on chromosome numbers and ploidy levels available for *Hieracium* species, information on the structure of their chromosome complexes is still missing. The cytogenetic characterization of diploid species seems particularly relevant in this respect, as they are considered to be the ancestors of apomictic allopolyploids, the origin of which in many cases has not been fully elucidated. To date, the results of more advanced karyotype analysis are limited to only a few diploid species: *Hieracium transylvanicum* (Ilnicki et al., 2010), *H. piloselloides* (Okada et al., 2011), *H. intybaceum*, *H. prenanthoides*, *H. alpinum* (Belyayev et al., 2018), and *H. vranceae* (Mráz et al., 2019).

The object of our study was *H. bracteolatum*, one of the few diploids whose number in the genus *Hieracium* is estimated at ca. 20 (Chrtek et al., 2004). In all examined specimens, the analysis of metaphase plates confirmed the somatic chromosome number typical of diploids, i.e., $2n = 18$ (Figs. 2a-c). The average total chromosome length varied from 2.78 to 4.19 μm and the size of the basal chromosome complex (x) was estimated as 30.80 μm . The morphology of the chromosome types was very similar: the chromosome complement consisted of eight pairs of metacentrics and only one pair of submetacentrics (pair 5) ordered according to their length (Table 1). The average arm length ratio (q/p) for metacentric chromosomes ranged from 1.21 to 1.55, for submetacentrics it was 1.8. Previous analyses conducted for other diploid species of *Hieracium*, e.g., *H. transylvanicum*, *H. alpinum*, *H. intybaceum*, *H. pre-*

anthoides also showed high karyotype symmetry, similarity of individual chromosome types and difficulty in distinguishing homologous pairs (Ilnicki et al., 2010; Belyayev et al., 2018). In *H. bracteolatum* the symmetry of the karyotype appeared to be even greater, compared to the mentioned species, in

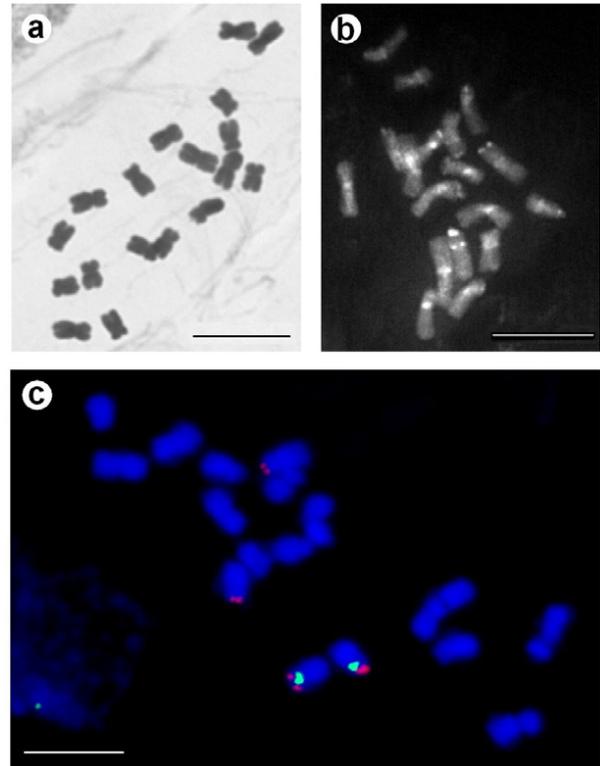


Fig. 2. Chromosomes of diploid *Hieracium bracteolatum*: (a) toluidine blue staining; (b) C-banding/DAPI staining; (c) rDNA-FISH: 45S rDNA (red) and 5S rDNA (green). Scale bars = 10 μm .

TABLE 1. Morphology of *Hieracium bracteolatum* chromosomes; chromosome types classified according to Levan et al., 1964.

	Long arm - <i>q</i> ($\mu\text{m} \pm \text{SD}$)	Short arm - <i>p</i> ($\mu\text{m} \pm \text{SD}$)	Total length ($\mu\text{m} \pm \text{SD}$)	Arm ratio (q/p)	Chromosome type
1	2,30 \pm 0,27	1,89 \pm 0,26	4,19 \pm 0,44	1,21	m
2	2,28 \pm 0,24	1,66 \pm 0,27	3,94 \pm 0,39	1,37	m
3	2,30 \pm 0,22	1,48 \pm 0,16	3,78 \pm 0,32	1,55	m
4	2,03 \pm 0,23	1,43 \pm 0,13	3,46 \pm 0,29	1,42	m
5	2,14 \pm 0,21	1,19 \pm 0,12	3,33 \pm 0,29	1,80	sm
6	1,92 \pm 0,19	1,34 \pm 0,12	3,26 \pm 0,25	1,43	m
7	1,88 \pm 0,22	1,23 \pm 0,15	3,11 \pm 0,30	1,53	m
8	1,71 \pm 0,17	1,24 \pm 0,11	2,95 \pm 0,21	1,37	m
9	1,67 \pm 0,13	1,11 \pm 0,16	2,78 \pm 0,22	1,50	m

SD – standard deviation; m – metacentric, sm – submetacentric.

which five to eight chromosome types with submedian centromere were described.

In this research, we made an attempt to obtain a banding pattern to distinguish *H. bracteolatum* chromosomes using the C-banding/DAPI method. This differential staining with fluorochrome DAPI (4',6-diamidino-2-phenylindole) revealed segments/dots of A-T rich heterochromatin observed mainly near the centromeres or terminally on the ends of chromosome arms, less frequently in the interstitial location. DAPI-positive bands were quite distinct and bright on a few chromosomes but on others they showed weak and sometimes diffuse fluorescence (Fig. 2b). In general, their distribution within individual chromosomes varied only slightly. C-banding/DAPI turned out to be a very useful method for identification of undistinguishable chromosomes, e.g., in *Humulus japonicus* (Grabowska-Joachimiak et al., 2011), but unfortunately, in the case of *Hieracium bracteolatum* it did not allow the determination of chromosome-specific markers. Nevertheless, it was possible to detect for the first time the occurrence of DAPI-positive segments, i.e., chromosome regions composed of tandem and A-T rich DNA repeats in this species. Cluster-distributed, genus specific satDNA elements (HintCI-18, HintCI-82, HintCI-160) derived from the next-generation sequencing (NGS) were previously described by Belyayev et al. (2018) within chromosomes of three relatively distantly related diploid *Hieracium* species. Fluorescent *in situ* hybridization with the probes of satDNAs showed differential, species-specific chromosomal distribution, but in all analyzed species the most abundant were pericentromeric 147-bp-long HintCI-18 sequences. Hybridization signals of a 297-bp HintCI-82 probe occurred in the terminal regions of some chromosomes and these of 415-bp HintCI-160 were located intercalary, terminally or near the centromeres, showing mainly dispersed distribution. Thanks to multi-FISH experiments with the HintCI probes in combination with rDNA probes, the authors obtained unique molecular-cytogenetic markers helpful for karyotyping in the genus *Hieracium*. Considering the relict nature of *H. bracteolatum*, in the future it would be worthwhile to verify whether this species has analogous sequences within DAPI-positive segments or whether they are of completely different molecular structure. No secondary constrictions or satellites were observed in any of the conventionally and differentially stained metaphases, probably due to the high degree of chromatin condensation, so it was not

possible to identify NOR chromosomes at this stage of the study. We used fluorescent *in situ* hybridization to detect chromosomal distribution of the 45S rDNA loci. In all analyzed metaphase plates hybridization sites were found on two chromosome pairs, at the end of the shorter arms (subterminally). In addition, the 5S rDNA sequences were also visible within one of these pairs. Moreover, they were present on the shorter arms, but in the pericentromeric region, in close proximity to the 45S rDNA (Fig. 2c). Such localization of the rDNA loci is consistent with results obtained for other diploids, i.e., *Hieracium alpinum*, *H. intybaceum* and *H. prenanthoides*, although in the latter species, the 45S rDNA clusters were identified within three chromosome pairs (Belyayev et al., 2018). Previous data on rDNA markers show that the number of 45S rDNA loci in *Hieracium* can vary, but its colocalization with 5S rDNA cluster on one pair of chromosomes seems to be a constant and characteristic feature of this genus (Ilnicki et al., 2010; Belyayev et al., 2018).

Hieracium species representing different degrees of ploidy (mainly tetraploids) have also been examined using FISH for the locus LOA (LOSS OF APOMEIOSIS), which is believed to control the initiation of apomixis in *H. praealtum* (Okada et al., 2011). Recently, Mráz et al. (2019) performed cytogenetic investigation involving FISH (rDNA, centromeric HintCI-18 loci) in a Romanian-origin endemic diploid *Hieracium vranceae*. Its karyotype was found to be very similar to the earlier studied *H. alpinum*, *H. intybaceum* (Belyayev et al., 2018) and *H. transylvanicum* (Ilnicki et al., 2010). Moreover, analysis with GISH (genomic *in situ* hybridization) showed that *H. vranceae* is the parental form for apomictic allotriploid *H. telekianum* (Mráz et al., 2019).

Our studies provide the first data on the karyotype structure in *H. bracteolatum*. Understanding the genome organization in the few diploid species that are considered basal evolutionary units in *Hieracium* seems crucial for explaining the origin of numerous allopolyploids and for clarifying intricate phylogenetic relationships in this interesting genus.

AUTHORS' CONTRIBUTION

AG-J – idea, interpretation of results, writing the original draft of manuscript; MŽ – slide preparation, conventional chromosome staining and

C-banding/DAPI; MŽ and AG-J – karyotype analysis; DK and MŽ - FISH; ZS – sampling and drafting of manuscript. The authors have declared that there is no conflict of interest.

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