Cytogenetic analysis of *Hieracium transylvanicum* (Asteraceae)

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Abstract — *Hieracium transylvanicum* is a relic species which occurs in refugial areas of south-eastern Europe**.** The structure of its karyotype, including determination of morphometric features of somatic metaphase chromosomes as well as the number and distribution of ribosomal DNA loci, was attempted using both classical chromosome staining and the FISH method with 25S rDNA and 5S rDNA probes.
The cytotypes under study were collected from eight sites and did not vary significantly in terms of their karyotype organisation. All 11 specimens had the same chromosome number, 2n = 2 x = 18, and 9 similar chromosomal types, whose total length ranged from 4.62 to 2.64 μm. The karyotype of the species is highly symmetrical. Two chromosomal types, 3 and 6, have a 45S rDNA locus in distal parts of their short arms. In addition, the short arm of type 6 has interstitial 5S rDNA sequences. No intraspecific variation in number and chromosomal distribution of ribosomal DNA loci was observed. The very uniform structure of the karyotype of *H. transylvanicum* possibly reflects the old phylogenetic age of this species.

Key words: Asteraceae, "autodiploid", fluorescence *in situ* hybridisation, *Hieracium transylvanicum*, karyotype, rDNA, sexual species.

INTRODUCTION

The genus *Hieracium* L. s. str. is dominated in Europe by apomictic tri- (2n = 3x = 27) and tetraploid (2n = 4x = 36) taxa of putative hybrid origin. Sexual diploids with 2n = 2x = 18 chromosomes represent as little as about 5% of species and their distribution is mainly restricted to refugial regions of southern Europe. One of the representatives is *H. transylvanicum* Heuff. that can be found in spruce and beech mountain forests in the Southern and Eastern Carpathians and in the western part of the Balkan Peninsula.

Karyological analyses of *H. transylvanicum* from various parts of its geographical range have revealed the diploid nature of this taxon (Rosenberg 1927; Chrtek 1996; Vladimirov 2000; Mráz 2003; Mráz and Szeląg 2004; Yurukova-Grancharova *et al.* 2006; Szeląg *et al.* 2007). However, all studies to date have been limited to the simple determination of the somatic chromosome number, which necessitates more detailed analyses of the *H. transylvanicum* karyotype. In the present study, cytogenetic investigation of 11 plants derived from eight separate Balkan and Carpathian populations was made (Table 1, Fig. 1), using both classical chromosome staining and fluorescence *in situ* hybridisation (FISH) with 25S ribosomal DNA (rDNA) and 5S rDNA probes. In this investigation the structure of the karyotype, including the chromosomal distribution of rDNA sites in genome of *H. transylvanicum*, was attempted.

MATERIALS AND METHODS

The living plants used in this study were collected from eight locations in Europe (Fig. 1). Detailed information on their origin is provided in Table 1.
Classical chromosome staining - Root tips were

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incubated overnight at 4°C in a saturated aqueous solution of α-bromonaphthalene and fixed in 1:3 acetic alcohol. The root tips were hydrolyzed with 1M HCl at 60°C and squashed in 45% acetic acid. The chromosomes were stained with a 0.1% aqueous solution of toluidine blue. The preparations were analyzed and photographed under a Nikon Optiphot 2 universal microscope equipped with a digital image acquisition system.

DNA labelling and FISH - The procedure followed the protocol described in detail by WOLNY and HASTEROK (2009) with minor modifications. In brief, the following DNA probes were used: (i) a 2.3 kb *ClaI* subclone representing the fragment of a 25S rDNA coding region of *Arabidopsis thaliana* (UNFRIED and GRUENDLER 1990) was labelled by nick translation with digoxigenin-11-dUTP (Roche, Indianapolis, IN, USA) and used to detect the 45S rDNA loci containing the genes encoding for 18S-5.8S-25S rRNA. (ii) pTa794 clone containing a 410 bp fragment of 5S rDNA unit isolated from wheat (GERLACH and DYER 1980) was labelled by PCR with tetramethyl-rhodamime-5-dUTP (Roche) and used to visualize 5S rDNA loci. The oligonucleotide primers sequence and conditions for the reaction were as described by HASTEROK *et al.* (2002).

The general conditions of the FISH procedure were as follows: slides were incubated in DNase-free RNase (100 µg/ml) in 2×SSC for 1 hr at 37°C, then washed in three changes of 2× saline sodium citrate (SSC) buffer for 15 min, post-fixed in 1% formaldehyde in PBS buffer followed by washes in 2×SSC for 15 min, dehydrated in an ethanol series (70, 90 and 100%) and air-dried. The DNA probes were mixed to a final concentration of 2.5-3.5 ng/µl of hybridisation mixture along with 50% deionised formamide, 20% dextran sulphate, 2×SSC, 0.5% SDS and salmon sperm blocking

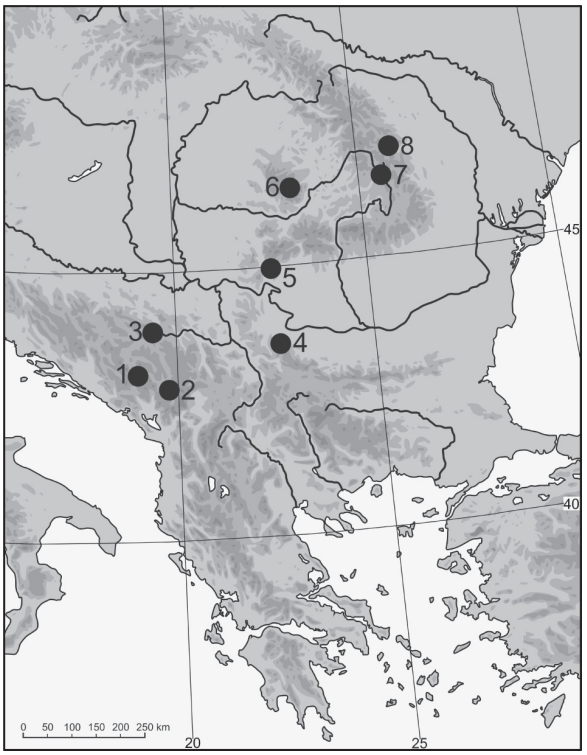

Fig. 1 — Geographical localization of eight collection sites of *Hieracium transylvanicum* used in this study.

TABLE 1 — Origin of *Hieracium transylvanicum* plant material used in the study.

No.	Country	Locality and habitat	No. of plants	Coordinates	2n
1	Montenegro	Durmitor Mts, Crno jezero Lake, *Picea abies* forest on limestone, 1440 m	1	43°08'59"N, 19°05'40"E	18
2	Montenegro	Bjelasica Mts, Biogradsko jezero Lake, *Fagus sylvatica* forest on limestone, 1100 m	2	42°53'55"N, 19°36'26"E	18
3	Serbia	Tara Mts, Mitrovac, *Fagus sylvatica-Picea abies* forest on acid soil, 1050 m	1	43°54'50"N, 19°25'28"E	18
4	Bulgaria	Western Stara Planina Mts, Chiprovska Mts, Mt. Kopren *Fagus sylvatica* forest, above village of Kopren, 1070 m	1	43°20'08"N, 22°51'50"E	18
5	Romania	Southern Carpathians, Mehedinti Mts, Mt. Domugled, *Fagus sylvatica* forest on limestone, 870 m	1	44°52'55"N, 22°26'23"E	18
6	Romania	Apuseni Mts, Muntele Mare Mts, Mt. Buscat, *Picea abies* forest on silicate, 1350 m	1	46°32'10"N, 23°10'08"E	18
7	Romania	Eastern Carpathians, Hargita Mts, Mt. Ciceu, *Picea abies* forest on silicate, 1300 m	2	46°23'33"N, 25°38'08"E	18
8	Romania	Eastern Carpathians, Ceahlău Mts, Mt. Toaca, *Picea abies* forest on limestone 1570 m	2	46°59'45"N, 25°55'15"E	18

DNA in 50-100× excess of labelled probes. The hybridisation mixture was pre-denatured (75°C for 10 min), applied to the chromosome preparations and denatured together at 75°C for 5 min in an Omnislide *in situ* hybridisation system (Hybaid, Basingstoke, UK) and then incubated overnight at 37°C in a humid chamber to allow renaturation. After hybridisation, slides were washed for 10 min in 15% deionised formamide in 0.1× SSC at 42°C, which is equivalent to 82% stringency, followed by several washes in 2× SSC. The digoxigenated probe was immunodetected according to standard protocols by antidigoxigenin antibodies conjugated with fluorescein isothiocyanate (FITC; Roche). After final dehydration, preparations were mounted and counterstained in Vectashield (Vector Laboratories, Burlingame, CA, USA) antifade buffer containing 2.5 μg/ml 4'-6-diamidino-2-phenylindole (DAPI; Serva). Microphotographs were taken using a Nikon Eclipse 80i epifluorescent microscope equipped with a monochromatic CCD camera and processed uniformly using Picture Publisher (Micrographx) software.

Chromosome measurements were made using a computer program CytoPlane ver. 1.2. Calculations, statistical analyses and schematic diagrams (idiograms) of chromosomes were made using the Mr Karyo ver. 3.07 (Tokarski & Joachimiak) software package. The chromosome nomenclature and types followed that of LEVAN *et al.* (1964).

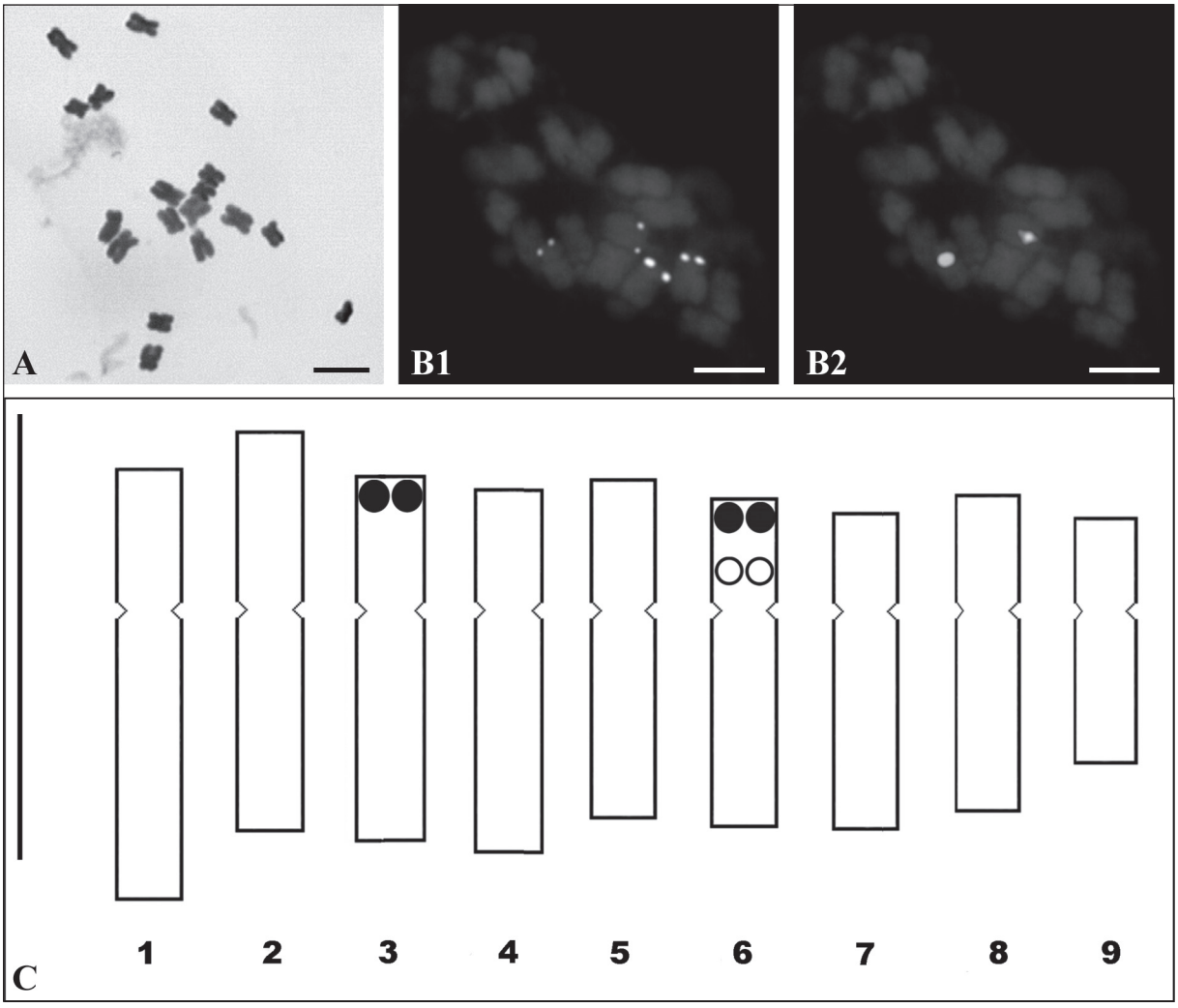

Fig. 2 — (A) Mitotic metaphase chromosomes of *Hieracium transylvanicum* (2n=18) stained with toluidine blue. (B1) FISH with 25S rDNA and (B2) 5S rDNA sequence as probes, chromosomes counterstained with DAPI. (C) idiogram showing total lengths of the chromosomes, centromere localizations and chromosomal positions of 25S rDNA (solid dark dots) and 5S rDNA (empty dots) sites. All bars: 5 μm.

RESULTS

All specimens of *H. transylvanicum*, collected from eight locations had the same somatic chromosome number of 2n = 2x = 18 (Table 1, Fig. 2A). The cytotypes under this study did not vary significantly in terms of their chromosome structure. An average karyotype was constructed basing on measurements of three selected metaphase plates subjected to FISH, representing two plants from the geographically distant Tara Mountains (2 metaphase plates) and Ceahlău Mountains (1 metaphase plate). In addition, morphometric analyses were performed on approximately 32 metaphase plates obtained from material of the nine other plants. This allowed detection of nine morphologically similar chromosomal types (Fig. 2C). In spite of high karyotype symmetry, it was possible to discriminate four types of marker chromosomes (1, 2, 7 and 9) which differed both in their total length and centromere position. Occasionally, two other chromosomal types (3 and 6) were observed, which sometimes had small secondary constrictions/satellites in distal parts of their short arms (like in studied *H. grovensianum* by FEDERICO and FIORINI 1996) that gave positive signals of hybridisation with the 25S rDNA probe (Fig. 2B1, Fig. 2C) indicating that these regions contain the genes encoding for 18S-5.8S-25S RNA. In the vicinity to the 45S rDNA locus of the chromosome type 6, more proximally distributed 5S rDNA sequences were found (Fig. 2B2, Fig. 2C). The three remaining chromosomal types (4, 5 and 8) did not reveal significant differences in their morphology. The total length of chromosomes in the karyotype ranged from 4.62 to 2.64 μm (Table 2).

DISCUSSION

Our study revealed that *H. transylvanicum* plants from eight different locations did not show any variation both in terms of chromosome number and morphology, thereby confirming the results of previous studies (ROSENBERG 1927; CHRTEK 1996; VLADIMIROV 2000; MRÁZ 2003; MRÁZ and SZELĄG 2004; YURUKOVA-GRANCHAROVA *et al.* 2006; SZELĄG *et al.* 2007). The genome was also highly uniform with regard to the number and chromosomal distribution of 45S rDNA and 5S rDNA sequences in plants derived from distant sites, such as the Tara Mountains in Serbia and the Ceahlău Mountains in Romania. The former sequences were found in two different chromosomal types (3 and 6), and in turn the 5S rDNA sequences were observed exclusively in chromosomal type 6. Such high stability of ribosomal DNA sites chromosomal distribution is usually observed in species with the low numbers of rDNA loci, e.g. in *Brachypodium distachyon* (DRAPER *et al.* 2001). In contrary, species with multiple rDNA sites, such as *Brassica rapa* and its many relatives, often show significant intraspecific polymorphism in this respect (HASTEROK *et al.* 2006).

The karyotype of the species showed high symmetry, consisting of rather uniform submeta- and metacentric chromosomes. Similar observations were made in some other species of *Hieracium* described by FEDERICO and FIORINI (1996); BICKNELL *et al.* (2000); COŞKUNCELEBI and HAYIRLIOĞLU-AYAZ (2006) and CASTRO *et al.* (2007). Based on the comparative results obtained from eight different sites, we conclude that the organisation of the *Hieracium* karyotype at the chromosomal level is very stable and shows

TABLE 2 — Average FISH karyotype of *H. transylvanicum* chromosomes based on the measurements of three selected metaphase plates. Centromere localization: m - median, sm - submedian.
* - chromosomal type with secondary constriction/satellite, sd - standard deviation.

Chromosomal type	Total length (μm)	Long arm (μm)	Short arm (μm)	Arm ratio	Centromere localization
1	4.62 (±0.54)	3.10 (±0.20)	1.52 (±0.22)	2.04	sm
2	4.29 (±0.27)	2.38 (±0.14)	1.91 (±0.15)	1.25	m
3*	3.91 (±0.63)	2.48 (±0.16)	1.43 (±0.18)	1.73	sm
4	3.90 (±0.49)	2.70 (±0.21)	1.20 (±0.17)	2.25	sm
5	3.64 (±0.39)	2.22 (±0.15)	1.42 (±0.16)	1.56	m
6*	3.52 (±0.36)	2.32 (±0.12)	1.20 (±0.13)	1.93	sm
7	3.40 (±0.34)	2.42 (±0.12)	0.98 (±0.14)	2.47	sm
8	3.39 (±0.42)	1.86 (±0.16)	1.53 (±0.66)	1.22	m
9	2.64 (±0.16)	1.64 (±0.15)	1.00 (±0.17)	1.64	m

that *H. transylvanicum* is an autodiploid species. This could possibly imply a very old phylogenetic age of this species and its distribution in refugial areas of southern Europe supports it. In this way, our studies are useful in terms of the determination of the geographical range of diploid *Hieracium* species as well as phylogenetic analyses.

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