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Analysis of *Hypericum* accessions by DNA fingerprinting and flow cytometry

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Abstract – *Hypericum perforatum*, *H. umbellatum*, *H. maculatum*, and *H. hircinum* accessions originating from botanical gardens across Europe were examined by flow cytometry and molecular markers. 2C DNA content of 17 *Hypericum perforatum* accessions (Hp) and the *H. perforatum* cultivar Topaz amounted to between 1.56 pg and 1.62 pg. In four Hp accessions some individual plants were found with a DNA content corresponding to 6Cx (2.34 - 2.39 pg). All plants of accession Hp8 showed a DNA content of 6Cx (2.41 pg). In root tips of Hp plants with an average DNA amount of 1.58 pg, 32 chromosomes were detected, corresponding to 2n = 4x. This is the first ploidy and/or DNA content report for *H. umbellatum, H. maculatum* and *H. hircinum. H. umbellatum* and *H. maculatum,* each contained 0.76 pg DNA and 16 chromosomes were counted. The 2C DNA content of *H. hircinum* was 1.00 pg with the best metaphase plate revealing 32 chromosomes. Additionally, a combined marker analysis, based on inter-simple sequence repeats (ISSR) and sequence related amplified polymorphism (SRAP), was conducted to gain a better understanding of diversity especially within the accessions of *H. perforatum*. A total of 27 (11 ISSR and 16 SRAP) primer combinations were screened, showing 699 bands, of which 661 were polymorphic. UPGMA clustering revealed that accessions from the same geographic area tended to be more closely related, while *H. maculatum* was grouped separately from all *H. perforatum* accessions. Both methods have shown similar sensitivities in detecting the genetic diversity of the analyzed genotypes. Our results may be useful for *Hypericum* breeding programs and the development of effective conservation strategies.

Keywords: chromosome number, DNA, genetic diversity, molecular markers, St. John's wort

Introduction

Hypericum L. (Hypericaceae) is a species-rich genus that colonized the temperate regions of the northern hemisphere and underwent rapid radiation during the Pleistocene (Scheriau et al. 2017). The genus consists of almost 500 species of shrubs, herbs and a few trees (Nürk and Blattner 2010), which were grouped into 36 sections (Robson 1981). Among *Hypericum* species only *H. perforatum* is widely used in medicine. So far, a limited number of species within the genus was studied, and the chemical compounds of approximately three quarters of *Hypericum* species have not been surveyed yet (Karioti and Bilia 2010). *Hypericum perforatum* extracts have multiple effects as an antidepressant, antiviral, antimicrobial and anti-inflammatory drug due to the main constituents, such as naphthodianthrones (hypericin and pseudohypericin), phloroglucinol derivatives (hyperforin), and flavonoids (quercetin, quercitrin, hyperoside and rutin).

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Because the substances are present only in small amounts but of great commercial interest, there have been strong efforts to enhance their production by biotechnological methods (Coste et al. 2011, Franklin et al. 2016). For this purpose, molecular characterization of native plants and/or accessions is necessary in order to identify the genotypes and to develop molecular markers associated with valuable traits.

In addition, the knowledge of the full range of ploidy variation is valuable for a proper management of genetic resources of *Hypericum* ssp. Extensive cytological investigations of *Hypericum perforatum* have made this species a model for examining aposporous apomixis (Barcaccia et al. 2007, Mártonfi and Mártonfiová 2011). This species has a basic chromosome number of 8, but mostly it is tetraploid (2n = 4x = 32), although diploids (2n = 2x = 16) and hexaploids (2n = 6x = 48) occur in natural populations (Matzk et al. 2001). Apomictic (polyploid) individuals can facultatively produce both sexual and variable apomictic seeds (Matzk et al. 2001, 2003, Barcaccia et al. 2007, Galla et al. 2011). Despite comprehensive studies about its modes of reproduction in relation to different ploidies, there is little knowledge about the distribution of different genome sizes and ploidies of individual plants within accessions, this distribution being an important feature especially for management of genetic resources. Furthermore, aneuploid individuals have been identified in Australian populations (2n-1 = 31) (Mayo and Langridge 2003). *H. perforatum* is hypothesized to hybridize easily with its sister *H. maculatum*, with mainly diploid populations (Brutovská et al. 2000; Barcaccia et al. 2007). Up to date, there have been reports of two diploid subspecies for *H. maculatum* (subsp. *maculatum* and subsp. *immaculatum*) and one tetraploid subspecies *H. maculatum* subsp. *obtusiusculum* (Koch et al. 2013). Regarding *H. hircinum*, this species was first reported as a possible pentaploid cytotype, with chromosome number varying between 40 (Loon and Jong 1978, Robson 1981) and 32 (Matzk et al. 2003, Castro and Rosselló 2006). To our knowledge, no data regarding *H. umbellatum* ploidy level and DNA content have been reported.

In the present paper, flow cytometry (FCM) was conducted to estimate the 2C DNA content of single plants of 17 accessions of *H. perforatum* (Hp) from botanical gardens all over Europe and the cultivar Topaz as well as Romanian accessions of wild species *H. maculatum* Crantz (Hm), *H. umbellatum* A. Kern. (Hu) and *H. hircinum* L. (Hh). The occurrence of various ploidies within the accessions was noted. For confirmation of the ploidy, chromosome counting was performed. Molecular characterization of *Hypericum* germplasm was accomplished by ISSR and SRAP markers.

Materials and methods

Plant material

Within this study, different accessions (populations) belonging to the genus *Hypericum* and covering four species namely *Hypericum perforatum* (Hp), *Hypericum maculatum* (Hm), *H. umbellatum* (Hu) and *H. hircinum* (Hh) from different botanical gardens of Europe were analyzed. In most of the accessions, seeds were from plants cultivated in botanical gardens (Hp1-8, Hp11-13, Hp15, Hm23, Hh24) but some of them were collected from plants from natural populations (Hp9-10, Hp14, Hp16-17, Hu21, Hm22) according to Tab. 1. Seeds were germinated in soil and plants grown in the greenhouse (at least 30 seeds/accession).

Flow cytometry analysis

Estimation of DNA content and ploidy by FCM was performed using fresh leaf material from young plants. Small amounts of leaf tissue of a sample and the internal standard were chopped together in 0.5 mL of Galbraith's buffer (Galbraith et al. 1983), supplemented with 0.5 mL Partec CyStain propidium iodide solution containing DNase-free RNase following the manufacturer's instructions (Partec) and filtered through a cell-strainer cap (BD Falcon™) with 35 μm pore size. Analyses were performed with a flow cytometer BD FACS Calibur (USA). For each sample, no fewer than 5000 particles were registered by 488 nm laser beam. For estimation of the nuclear DNA content, *Raphanus sativus* L. was used as an internal standard (2C = 1.11 pg; Doležel et al. 1992). Analyses were carried out for at least

Tab. 1. Accessions of *Hypericum* spp. studied and their origin (species code are given in parenthesis, * – seeds collected from plants cultivated in a botanical garden, ** – seeds collected from natural populations).

Accession name	Origin	Seeds / DNA source
H. perforatum (Hp) 1	Germany	Botanical Garden Ulm
Hp2*	Germany	Botanical Garden Frankfurt
Hp3*	Germany	Botanical Garden Regensburg
Hp4*	Germany	Humboldt University Berlin
Hp5*	Germany	Botanical Garden Constance
Hp6*	Germany	Botanical Garden Hamburg
Hp7*	Switzerland	Botanical Garden St. Gallen
Hp8*	Austria	Botanical Garden Salzburg
Hp9**	Austria	Botanical Garden Graz
Hp10**	France	Botanical Garden Nancy
Hp11*	France	Botanical Garden Ville de Renne
Hp12*	France	Botanical Garden Talence
Hp13*	Poland	Botanical Garden Wrocław
Hp14**	Norway	Botanical Garden Oslo
Hp15*	Italy	Botanical Garden Trento
Hp16**	Italy	Botanical Garden Siena
Hp17**	Estonia	Läänemaa, Hort Bot Tartu University
Hp cultivar 'Topaz'	Germany	Seed provider, Fürstenwalde
H. umbellatum (Hu21)**	Romania	Gilău
H. maculatum (Hm22)**	Romania	Piatra Craiului
H. maculatum (Hm23)*	Romania	Botanical Garden Cluj-Napoca
H. hircinum (Hh24)*	Romania	Botanical Garden Cluj-Napoca

five randomly selected individuals per accession and three repetitions for each individual. For accessions with differing cytotypes up to 30 individual plants were tested (Tab. 2). Data evaluation was accomplished with BD software CellQuest™Pro. Nuclear DNA content was calculated using the linear relationship between the ratio of the 2C peak positions of the target species/internal standard on the area histogram of fluorescence intensities. The statistics were carried out using the Real Statistics Resource Pack Version 4.9 for Microsoft Excel.

Squash preparations of root tip cells

Root tips were harvested from greenhouse plants and pretreated with 8-hydroxyquinoline for 2.5 hours at room temperature and fixed overnight in ethanol: glacial acetic acid (3:1 v/v) at 4 °C. The fixed root tips were transferred to 70% ethanol and stored at 4 °C. For further investigation fixed root tips were washed in distilled water for 10 min and then incubated at 37 °C for 45 min in an enzyme mix consisting of 4% celullase, 1% pectolyase and 45% acetic acid, pH 4.0. After being washed with distilled water, the tips were squashed on the slide in 45% acetic acid. If under a bright microscope in phase contrast metaphase chromosomes were observed, the slide was frozen for at least 15 min at -80 °C and the cover slip was blown off. After an air drying for 10 min or longer 15 µl DAPI VECTASHIELD® antifade mounting medium with DAPI (4', 6-diamidino-2-phenylindol, Vector Laboratories) was added. The chromosomes were detected and photographed in fluorescent light (microscope Axioimager Z1 with CCD-camera Axiocam, Zeiss). The image analysis was carried out with the software program Isis (MetaSystems, Germany).

ISSR and SRAP markers analysis

Marker analysis was conducted with 90 individuals (5 individuals/available accession), a representative sub-set of accessions and individuals screened by flow cytometry. Genomic DNA was isolated from young leaves of plants grown

Tab. 2. DNA content of accessions of *Hypericum perforatum* (Hp), *H. umbellatum* (Hu), *H. maculatum* (Hm), *H. hircinum* (Hh) assessed with internal standard *Raphanus sativus* (2C = 1.11 pg). -1, -2: plants belonging to the same accession but with different DNA content.

Accession	№ of plants investigated	№ of measurements	DNA content Mean (pg)	Standard Deviation	Ploidy	1Cx Content (pg)
Hp1	5	15	1.58	0.03	4x	0.40
Hp2	15	35	1.57	0.04	4x	0.39
Hp3-1	15	33	1.58	0.03	4x	0.40
Hp3-2	1	3	1.99	0.02	?	?
Hp4	5	15	1.56	0.01	4x	0.39
Hp5	5	15	1.59	0.02	4x	0.40
Hp6-1	24	30	1.57	0.03	4x	0.39
Hp6-2	2	7	2.38	0.05	6x	0.40
Hp7-1	30	38	1.56	0.02	4x	0.39
Hp7-2	5	10	2.34	0.02	6x	0.39
Hp8	5	12	2.41	0.07	6x	0.40
Hp9-1	25	33	1.58	0.03	4x	0.40
Hp9-2	1	3	2.37	0.03	6x	0.40
Hp10-1	23	44	1.57	0.02	4x	0.39
Hp10-2	2	5	2.39	0.05	6x	0.40
Hp11	5	15	1.59	0.03	4x	0.40
Hp12	5	14	1.62	0.01	4x	0.40
Hp13	5	15	1.58	0.01	4x	0.40
Hp14	5	15	1.57	0.02	4x	0.39
Hp15	5	15	1.59	0.02	4x	0.40
Hp16	5	15	1.59	0.01	4x	0.40
Hp17	5	15	1.59	0.02	4x	0.40
Hp 'Topaz'	6	7	1.58	0.01	4x	0.40
Hp (average for 4x)	188	369	1.58	0.01		0.40
Hp (average for 6x)	15	37	2.38	0.02		0.40
Other species						
Hu21	5	13	0.76	0.02	2x	0.38
Hm22	10	14	0.76	0.03	2x	0.38
Hm23	5	14	0.76	0.01	2x	0.38
Hh24	11	14	1.00	0.04	4x	0.25

under standard greenhouse conditions, by using the CTAB method described by Doyle and Doyle (1987). DNA concentration was estimated using a UV-Vis spectral photometer Nanodrop 8000. ISSR (Inter Simple Sequence Repeats) amplification was performed with 11 primers (Rostami-Ahmadvandi et al. 2013). For SRAP (Sequence Related Amplified Polymorphism) analysis, sixteen primer combinations (Li and Quiros 2001) were used. Primer sequences are given in Tab. 2. PCR amplification was performed in a 0.2 mL tube containing 12.5 µL 2x DreamTaq Green PCR master mix (Thermo Fisher Scientific, USA), 10.25 µL nuclease-free water (Lonza, Switzerland), 25 pmol of each primer (Eurogentec, Belgium) and 5 ng of genomic DNA in a final volume of 25 µL. For ISSR analysis the GeneAmp PCR system 9700 (Applied Biosystems, Forster City, USA) was programmed as follows: 94 °C for 4 min, 35 cycles of 94 °C for 30 sec, 46 °C for 30 sec and 72 °C for 55 sec followed by a final elongation step at 72 °C for 5 min. For SRAP analysis, the following program was used: 94 °C for 4 min, 5 cycles of 94 °C for 30 sec, 35 °C for 30 sec, and 72 °C for 55 sec followed by 30 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 55 sec, and a final elongation at 72 °C for 5 min. Amplification products were separated in 1.5% w/v agarose (Cleaver Scientific, United Kingdom) gel in 1×TBE buffer (Lonza, Switzerland) and stained with 0.5 µg/mL ethidium bromide (Thermo Fisher Scientific, USA).

ISSR and SRAP patterns were assessed as dominant markers. Band patterns for both marker systems were recorded in 1/0 matrices to determine the level of genetic similarity between the different accessions on the basis of Jaccard's coefficient (Jaccard 1908). The resulting matrix of similarity was analyzed by the unweighted pairgroup method with arithmetic mean (UPGMA) and the dendrogram was obtained using MultiVariate Statistical Package 3.21 (Kovach 2007). Shannon's information index (I) (Shannon and Weaver 1949) and expected heterozygosity (He) were calculated, using GenAlEx software version 6.5 (Peakall and Smouse 2012). The polymorphism information content (PIC) value of each individual locus was calculated according to Sehgal et al. (2009) as:

$$PIC_j = 1 - \Sigma pi^2_{n=1}$$

Where *i* is the ith allele of the jth marker, *n* is the number of alleles at the jth marker and p is the allele frequency.

Resolving power (Rp) for the individual marker system was bases on the distribution of detected bands within the sampled clones and was calculated based on the formula described by Prevost and Wilkinson (1999):

$$Rp = \Sigma Ib$$

Where Ib (informativeness) is 1- [2 x |0.5-*p*|] and *p* is the ratio of present bands among the analyzed accessions.

Results

Estimation of nuclear DNA content and determination of ploidy

The flow cytometric measurements consistently showed a high reproducibility in repeated measurements of the same plant despite the presence of numerous secondary metabo-

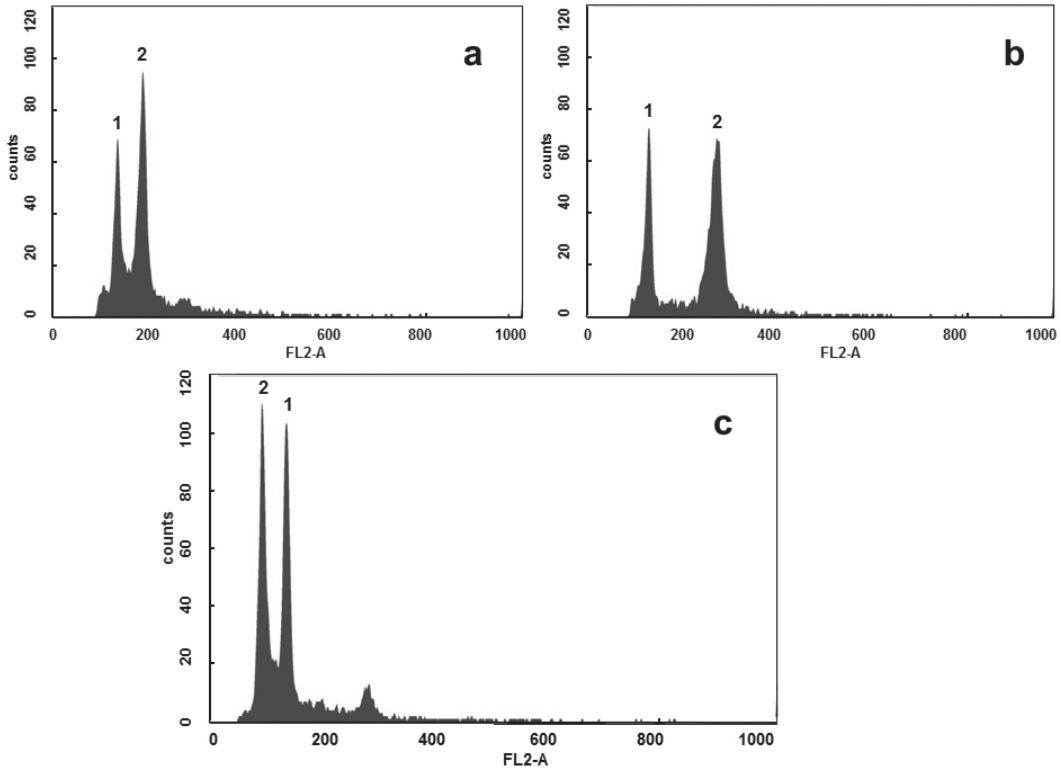

Fig. 1. Flow cytometric histograms (1: 2C Peak of internal standard *Raphanus sativus*, 1.11 pg). a – *Hypericum perforatum* Hp7-1: 2: 2C = 4Cx peak, 1.55 pg, b – Hp7-2: 2: 2C = 6Cx peak, 2.34 pg, c – *H. maculatum* Hm23: 2: 2C = 2Cx peak, 0.76 pg.

Tab. 3. SRAP (sequence related amplified polymorphism) and ISSR (inter-simple sequence repeats) primers used for amplification with their respective codes and nucleotide sequences.

Primer	Sequence	Primer	Sequence
SRAP		ISSR	
SRP2	Fw 5'-TGAGTCCAAACCGGAGC-3' Rv 5'-GACTGCGTACGAATTAAT-3'	A	$(GACA)_3RT$
SRP5	Fw 5'-TGAGTCCAAACCGGAAG-3' Rv 5'-GACTGCGTACGAATTAAT-3'	C	$(GACAC)_2$
SRP6	Fw 5'-TGAGTCCAAACCGGATA-3' Rv 5'-GACTGCGTACGAATTTGC-3'	UBC808	$(AG)_8C$
SRP11	Fw 5'-TGAGTCCAAACCGGATA-3' Rv 5'-GACTGCGTACGAATTGAC-3'	UBC809	$(AG)_8G$
SRP12	Fw 5'-TGAGTCCAAACCGGAGC-3' Rv 5'-GACTGCGTACGAATTGAC-3'	UBC811	$(GA)_8C$
SRP13	Fw 5'-TGAGTCCAAACCGGAAT-3' Rv 5'-GACTGCGTACGAATTGAC-3'	UBC112	$(GACA)_4$
SRP14	Fw 5'-TGAGTCCAAACCGGACC-3' Rv 5'-GACTGCGTACGAATTGAC-3'	UBC818	$(CA)_8G$
SRP15	Fw 5'-TGAGTCCAAACCGGAAG-3' Rv 5'-GACTGCGTACGAATTGAC-3'	UBC855	$(AC)_8YT$
SRP17	Fw 5'-TGAGTCCAAACCGGAGC-3' Rv 5'-GACTGCGTACGAATTTGA-3'	UBC856	$(ACAC)_4YG$
SRP20	Fw 5'-TGAGTCCAAACCGGAAG-3' Rv 5'-GACTGCGTACGAATTTGA-3'	UBC857	$(AC)_8T$
SRP25	Fw 5'-TGAGTCCAAACCGGAAG-3' Rv 5'-GACTGCGTACGAATTAAC-3'	UBC873	$(ATG)_6$
SRP26	Fw 5'-TGAGTCCAAACCGGATA-3' Rv 5'-GACTGCGTACGAATTGCA-3'		
SRP27	Fw 5'-TGAGTCCAAACCGGAGC-3' Rv 5'-GACTGCGTACGAATTGCA-3'		
SRP28	Fw 5'-TGAGTCCAAACCGGAAT-3' Rv 5'-GACTGCGTACGAATTGCA-3'		
SRP29	Fw 5'-TGAGTCCAAACCGGACC-3' Rv 5'-GACTGCGTACGAATTGCA-3'		
SRP30	Fw 5'-TGAGTCCAAACCGGAAG-3' Rv 5'-GACTGCGTACGAATTGCA-3'		

lites, which often influence the peak performance. The CV (coefficient of variation) of histogram peaks was below 5.0 in each case. *Raphanus sativus* served as an internal standard for genome size estimation. Both 2C peaks from internal standard and *Hypericum* sample were well separated from one another (Fig. 1). The genome size of 17 Hp accessions and the cultivar Topaz of the tetraploid genotypes amounted to between 1.56 pg and 1.62 pg (Tab. 2, Fig. 1a) with an average genome size of 2C = 4Cx = 1.58 pg. The *Hypericum* accessions were obtained from various European botanical gardens and from Romanian regions (Tab. 1). The accession Hp8 from the Salzburg botanical garden, Austria, revealed for five tested plants a hexaploid cytotype with a DNA content of 6Cx = 2.41 pg (Tab. 2). Besides tetraploid plants we found in Hp6, Hp7, Hp9 and Hp10 at least one plant which showed a genome size corresponding to the hexaploid chromosome level (Fig. 1a, b), taking it into account that the 1Cx DNA content for all Hp accessions amounted to 0.40 pg. However, one exception was noticed. In the Hp3 from the botanical garden in Regensburg, Germany, one plant with a genome size of 1.99 pg was observed. Only speculation could be offered about the nature of this cytotype.

For both Romanian *H. maculatum* accessions as well as for *H. umbellatum* a small genome size with 0.76 pg was estimated (Fig. 1c). The genome size of *H. hircinum* was 1.00 pg (Tab. 2). Thus, the 1Cx value of *H. hircinum* differed considerably from the others and was only 0.25 pg.

Chromosome number

For correct assignment of genome size to ploidy, the numbers of metaphase chromosomes were counted in stained root tips. The chromosomes of *Hypericum* are very small and morphologically similar, making chromosome counting difficult. In addition, the plant tissue digestion posed problems with regard to achieving well-spread chromosome plates. The unsatisfactory quality in connection

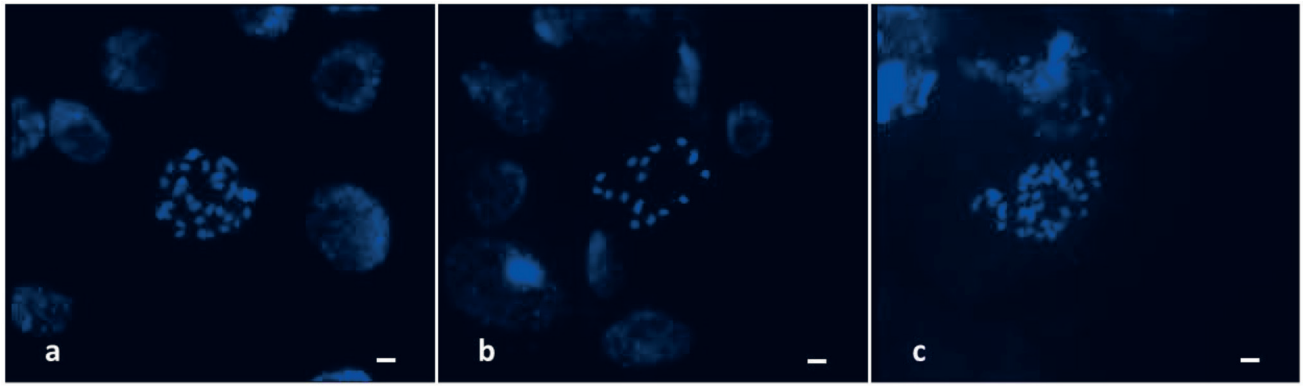

Fig. 2. Chromosomes in root tips stained with DAPI. a – *Hypericum perforatum* Hp7, 32 chromosomes, b – *H. maculatum* Hm22, 16 chromosomes, c – *H. hircinum* Hh24, 32 chromosomes. Scale bar: 5 μm.

with low mitotic index resulted in a fluctuating number of chromosomes being counted. In the accessions Hp3, Hp4, Hp7, and Hp9 we noticed 30 – 34 chromosomes, mainly 32 (Fig. 2a, Tab. 4). In *H. umbellatum* and *H. maculatum* 16 chromosomes were detected (Fig. 2b, Tab. 4). In squash preparations of *H. hircinum* 30 – 40 chromosomes were found, in the best preparations 32 chromosomes (Fig. 2c, Tab. 4). Especially in *H. hircinum,* chromosomes often so stuck together that even at different focal levels an unambiguous assessment of the chromosomes was problematic.

ISSR and SRAP marker analysis

In all, 699 amplified DNA bands were scored using 11 ISSR primers (298 markers) and 16 SRAP primers (401 markers) for the 17 *H. perforatum* accessions and one *H. maculatum* accession (Hm22).

The overall number of detected individual bands per primer ranged from 9 (SRP17) to 53 (SRP30), with an average of 27.1/25.1 (ISSR/SRAP) (Tab. 5). The combined analysis of ISSR and SRAP markers revealed a total of 661 (94.6%) polymorphic bands. The marker performance was estimated by two parameters: PIC value and resolving power (RP). ISSR primers and SRAP primer combinations showed the same mean PIC value (0.38). The highest PIC value was determined for primers UBC808, UBC809, UBC857, UBC873 and SRP26 (0.49). The resolving power (RP) of primers tested varied between 0.44 (UBC818/ SRP29) and 1.69 (UBC112). The mean values for Shannon's information index (I) and expected heterozygosity (He) were 0.39/0.25 for ISSR primers, and 0.44/0.29 for SRAP markers (Tab. 5).

Combined ISSR-SRAP UPGMA revealed two major clusters (Fig. 3): Cluster I comprising all *H. perforatum* accessions and cluster II represented by *H. maculatum* (Hm22) as a clearly separated outgroup. Within cluster I we observed 6 subclusters: Hp1 and Hp2 from Germany; Hp7 (Switzerland) and Hp9 (Austria); Hp13 (Poland) and Hp14 (Norway); Hp5 and Hp6 from Germany. French Hp10 and Hp11 and Italian Hp16 and Hp15 grouped with Hp17 from Estonia. Four *H. perforatum* accessions, Hp3 and Hp4 from Germany as well as Hp8 from Austria and Hp12 from France were separated in individual branches from the other genotypes. *H. maculatum* is clearly distinguished from all *H. perforatum* accessions.

Discussion

Nuclear DNA content and ploidy

The key to a successful breeding program is a better understanding of the extent and nature of genetic diversity present in wild, conserved and/or actively utilized germplasm of various species. There is a broad interest in gaining a better understanding of diversity within *Hypericum* species, especially in *H. perforatum*, due to its pharmaceutical importance but also for its remarkable evolutionary and adaptive capacities. Therefore, we employed FCM for analysis of ploidy and genome size as well as two types of molecular markers (ISSR and SRAP) to reveal the genetic diversity and relationships among several *Hypericum* accessions.

FCM is a powerful tool for genome size estimation and ploidy determination. With all the advantages and possi-

Tab. 4. Chromosome number in root tips of different *Hypericum* accessions (Hp - *H. perforatum*, Hu – *H. umbellatum*, Hm – *H. maculatum*, Hh – *H. hircinum*).

Accession	№ of chromosome counts	Counted chromosomes
Hp3	3	31 - 32
Hp4	2	30 - 31
Hp7	23	28 - 34, of which 10 times 32 chromosomes
Hp9	1	32 - 34
Hu21	11	14 - 16
Hm22	9	13 - 16
Hh24	13	30 - 40, of which 5 times 32 chromosomes

Tab. 5. Estimation of the genetic diversity of 18 *Hypericum* accessions. TNB – total number of bands, NPB – number of polymorphic bands, RP – resolving power-average, PIC – polymorphic information content, I – Shannon's Information Index, He – expected heterozygosity, ISSR – inter-simple sequence repeats, SRAP – sequence related amplified polymorphism.

Primer	Detected amplification products		% of polymorphic loci	RP	PIC	I	He
	TNB	NPB					
ISSR							
A	23	21	91.3	0.99	0.45	0.43	0.28
C	13	13	100.0	0.50	0.25	0.33	0.20
UBC808	24	20	83.3	1.38	0.49	0.49	0.33
UBC809	21	18	85.7	1.25	0.49	0.44	0.29
UBC811	46	46	100.0	0.58	0.27	0.41	0.25
UBC112	20	8	40.0	1.69	0.35	0.23	0.16
UBC818	31	31	100.0	0.44	0.21	0.33	0.20
UBC855	42	42	100.0	0.51	0.25	0.33	0.20
UBC856	24	22	91.7	0.98	0.45	0.42	0.27
UBC857	23	21	91.3	1.21	0.49	0.47	0.31
UBC873	31	27	87.1	1.27	0.49	0.45	0.30
Total	298	269	90.3	-	-	-	-
Average	27.1	24.5	-	0.98	0.38	0.39	0.25
SRAP							
SRP2	22	22	100.0	0.92	0.42	0.47	0.31
SRP5	22	22	100.0	0.76	0.35	0.45	0.29
SRP6	25	23	92.0	0.81	0.39	0.42	0.27
SRP11	21	21	100.0	0.78	0.37	0.43	0.28
SRP12	18	18	100.0	0.97	0.42	0.54	0.37
SRP13	26	26	100.0	0.88	0.41	0.46	0.30
SRP14	26	26	100.0	0.90	0.41	0.47	0.31
SRP15	16	16	100.0	1.08	0.45	0.55	0.38
SRP17	9	9	100.0	0.68	0.36	0.31	0.19
SRP20	21	20	95.2	0.89	0.41	0.47	0.31
SRP25	25	25	100.0	1.09	0.46	0.52	0.35
SRP26	22	18	81.8	1.37	0.49	0.48	0.33
SRP27	30	29	96.7	1.01	0.44	0.52	0.35
SRP28	30	30	100.0	0.46	0.22	0.33	0.20
SRP29	35	35	100.0	0.44	0.21	0.32	0.19
SRP30	53	52	98.1	0.58	0.29	0.34	0.21
Total	401	392	97.8	-	-	-	-
Average	25.1	24.5	-	0.85	0.38	0.44	0.29
ISSR + SRAP							
Total	699	661	94.6	-	-	-	-
Average	25.9	24.5	-	0.90	0.38	0.42	0.28

bilities of FCM, however, it must be taken into account that the results are always expressed in relation to a known standard. For genome size estimation (pg value) a plant with an already defined DNA content as internal standard should be measured together with the sample plant in the staining solution. For ploidy estimation, plants with a cytologically verified chromosome number within the same species serve as standard, providing that different ploidy degrees are in linear dependency.

For 17 *H. perforatum* accessions from different botanical gardens across Europe and the cultivar Topaz, the genome size was on average 1.58 pg ranging from 1.56 to 1.62

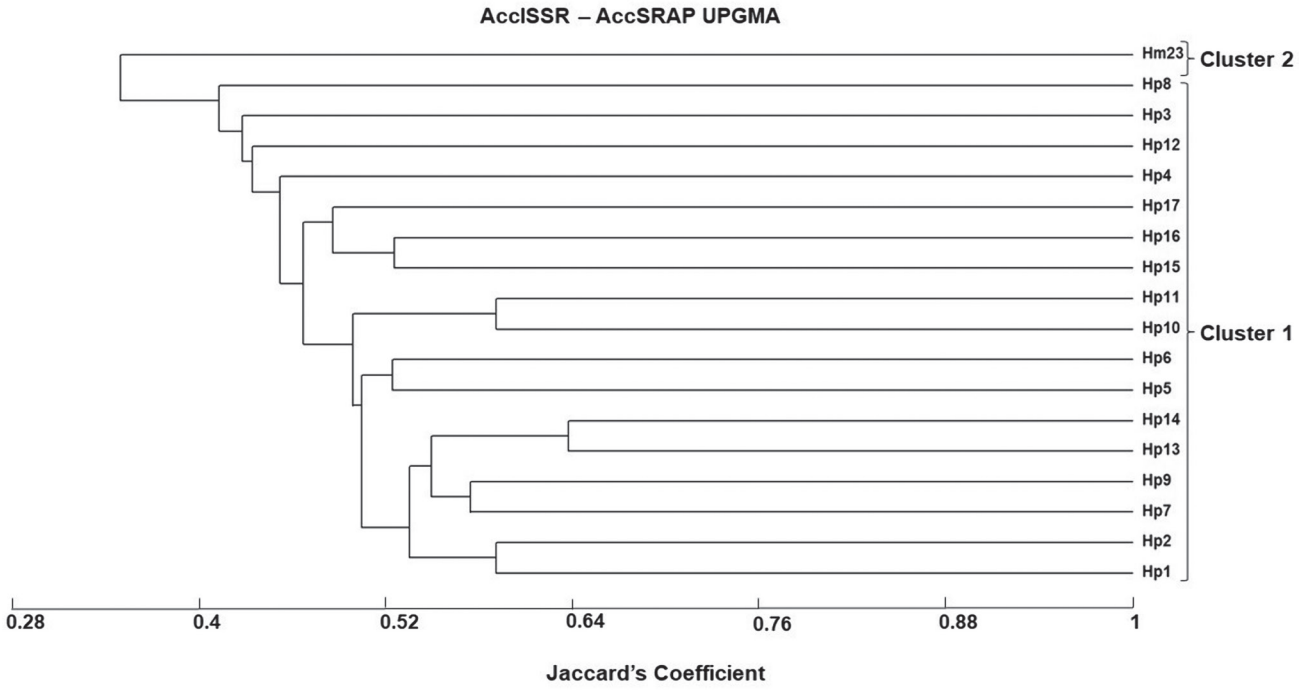

Fig. 3. UPGMA dendrogram generated by Jaccard's similarity coefficients showing relationships among 18 *Hypericum* accessions based on combined data from ISSR-SRAP (inter-simple sequence repeats analysis – sequence related amplified polymorphism). The samples are labeled with the codes listed in Tab. 1.

pg in the different tetraploid accessions. Chromosome counting in root tips of different *Hypericum* plants with the corresponding genome size of 1.58 pg revealed 2n = 4x = 32 chromosomes. These results are in agreement with Temsch et al. (2010) (2C = 1.59 pg), and Alan et al. (2015) (2C = 1.50 pg). The slight differences result from employment of different standards, extraction / staining buffers and flow cytometers (Doležel and Bartoš 2005) but the use of various accessions and the physiological state of the plant material also have an influence on the measurement.

Matzk et al. (2003) developed the flow cytometric seed screen (FCSS), boosted the investigation of apomixes of *Hypericum* and broaden the knowledge about apomictic pathways in general. However, reports about the germination capacity of the differently developed seeds and their contribution to plant populations are very limited. Thought, such information would have an impact on germplasm management and preservation strategies of rare natural populations. Mixed cytotypes in populations could provide novel traits for crop development and therefore the seed samples in gene banks should be adapted to that situation. *Hypericum perforatum* is predominately tetraploid (Matzk et al. 2003, Galla et al. 2011). Barcaccia et al. (2007) describe wild populations of *H. perforatum* in more detail as populations with diploid and polyploid (mainly tetraploid) plants. Savaş Tuna et al. (2017) analyzed three seedlings from 39 Hp accessions each from different regions of Turkey and revealed a nuclear DNA content between 0.8 – 2.57 pg. Of the 39 accessions, one was diploid, 5 hexaploid and 33 tetraploid but no ploidy variation was noticed inside the accessions. Perhaps the test of only three seedlings per accession is not sufficient to provide reliable findings. Here we present flow cytometric DNA size determination of plants of 17 belonging to Hp accessions and the cultivar Topaz. Tetraploidy was revealed with only one exception: all plants from Hp8 from Salzburg have shown a DNA size of 2.41 pg, corresponding to the hexaploid level. This uniformity could be explained by the origin of seeds in plants cultivated in botanical garden, most probably the collection being not very diverse. In four accessions (Hp6-7 and Hp9-10), there were mixed cytotypes (4x + 6x). This is an interesting situation, as Hp 6-7 seeds were collected from plants cultivated in botanical gardens and Hp 9-10 belong to plants from natural populations. Similar results were found in three *H. perforatum* accessions (Qu et al. 2010). Despite the diverse embryo and endosperm ratios observed in seeds, Qu et al. (2010) found only tetraploids and hexaploids in seedling populations with tetraploids constituting 87 - 97% but a complete hexaploid population was not detected. Among the accessions presented here the accession Hp8 shows only hexaploid plants indicating that such accessions at least with a high proportion of hexaploid plants could exist and depend on the seed source. Moreover, in accession Hp3 a single plant was found with 1.99 pg. Since it does not fit the 1C content of 0.40 pg, one can assume that it is an aneuploid plant reflecting additionally the high plasticity of the *H. perforatum* genome, even if Hp3 comes from plants that are cultivated in a botanical garden.

To our knowledge we report for the first time the genome size of *H. maculatum* (two accessions, one from a natural habitat and one cultivated in a botanical garden), *H. umbellatum* (from natural habitat) (0.76 pg each) and *H.*

hircinum (from botanical garden) (1.00 pg). The first three accessions are native to Romania. Hence, the DNA size of *H. hircinum* of 1.0 pg is between the estimated DNA size for the diploid species *H. maculatum* und *H. umbellatum* (0.76 pg) and the tetraploid *H. perforatum* (1.58 pg). The genome size for *H. maculatum* and *H. umbellatum* corresponds to the chromosome number 2n = 2x = 16. For *H. hircinum* Loon and Jong (1978) published a chromosome number 2n = 40. They explained the high chromosome number of 40 as a possible pentaploid cytotype. Castro and Rosselló (2006) found 2n = 32 in *H. hircinum* subsp. *cambessedesii*, an endemic plant from the Balearic Islands. The *H. hircinium* accession investigated in the present paper was provided by the Alexandru Borza Botanical Garden from Cluj-Napoca, Romania. In this accession, we observed 30 - 40 chromosomes; in the best metaphase plates 32, which is in agreement with the findings of Castro and Rosselló (2006). The result was surprising because *H. hircinum* has a much lower DNA size than *H. perforatum* (1.58 pg) although it also has 32 chromosomes. This fact underlines the high variability of the genus *Hypericum*.

Genetic polymorphism

Characterization of *Hypericum* species by different molecular marker types has been performed over the years (Corral et al. 2011, He and Wang 2013). ISSR and SRAP markers were chosen due their advantages: cost efficiency, informativeness, versatility and reproducibility. We employed a set of 11 ISSR primers and 16 SRAP primer combinations to assess the genetic diversity among one Hm and 17 Hp accessions. ISSR and SRAP markers have proved to be highly polymorphic. The average number of polymorphic bands per primer was the same (24) for both types of markers. Several studies report that when several marker types are used, they can be complementary tools for genetic diversity analysis, because they can be used to amplify different regions of the genome (Chen et al. 2013). Our study revealed a significantly higher rate of polymorphism in the analyzed *Hypericum* germplasm for both SRAP (97.8%) and ISSR (90.2%) markers than previously reported (He and Wang 2013). This might be explained by a larger sampling area with very different Hp accessions from botanical gardens in Europe and native wild accessions from Romania. The two marker systems used in our study revealed close degrees of resolution (Tab. 5).

Species cluster analyses based on combined ISSR-SRAP data (Fig. 3) indicate that *H. maculatum* is closely related to some *H. perforatum* accessions. These taxa, belonging to section *Hypericum* "core *Hypericum*" (Nürk 2011), were proven to be in close contact and apparently hybridize frequently, which might explain the sympatric occurrence of morphologically similar taxa (Robson 2003, Koch et al. 2013). Thus, according to Brutovská et al. (2000), *H. perforatum* probably originates from autopolyploidization of an ancestor closely related to diploid *H. maculatum*, while Robson (2003) regards *H. perforatum* as an allopolyploid, derived from a cross between *H. maculatum* subsp. *immaculatum* and *H. attenuatum*. This close relationship between the two species was also supported by later studies based on different marker types, such as RFLP and cpDNA markers, as well as phylogenetic studies using nuclear internal transcribed spacer (ITS) sequences (Pilepić et al. 2011). However, it was recently implied that *H. perforatum* is not of hybrid origin (Koch et al. 2013). The authors suggest that *H. perforatum* has a single evolutionary origin arising from independent and recurrent polyploidization of two different ancestral gene pools along with occurrence of substantial gene flow within and between *H. perforatum* and *H. maculatum*.

Regarding the cluster analysis of *H. perforatum* accessions, we have noticed a partially regional-based relationship. Some accessions from the same regions shared similar genotypes and ploidy levels (Hp1 and Hp2 from Germany; HP15 and HP16 from Italy), and the same was noticed for accessions from different geographical locations (HP9 from Austria and HP7 from Switzerland; HP13 from Poland and HP14 from Norway) (Tab. 1, 3; Fig. 3). Moreover, mixed ploidy accessions (4x and 6x) (Hp5 and Hp6; Hp9 and Hp7; Hp10 and Hp11) shared similar genotypes, while the complete hexaploid accession HP8 from Austria is clearly of different genetic origin from that of the tetraploid Hp9 accession from the same country (Tab. 1, 3; Fig. 3). This endorses the high plasticity in ploidy and reproductive system of *H. perforatum*, regardless of geographic origin (Koch et al. 2013). Differences among *H. perforatum* genotypes were reported in different *H. perforatum* wild populations and landraces as confirmed by molecular, morphometric and cytogenetic analyses (He and Wang 2013, Morshedloo et al. 2014). The high genetic diversity exhibited by our analysis might be explained by the diverse mating systems of *H. perforatum* from sexual cross to apomixis.

Conclusions

We report the 2C DNA content of 17 *H. perforatum* accessions from different botanical gardens in Europe. 2C DNA content of *H. perforatum* found in our study was 1.58 pg. The tetraploid degree of the plants was confirmed by chromosome counting. FCM is a fast and reliable method for screening the variability inside a *Hypericum* accession concerning ploidy distribution. Besides the tetraploids, few hexaploid plants and one putative aneuploid plant were found in the *H. perforatum* accessions, independent of the origin of the seed. Mixed cytotypes (4x+6x) were identified in accessions from natural populations and cultivated in botanical gardens as well. One *H. perforatum* accession was characterized as hexaploid. The genome size of *H. maculatum, H. umbellatum* and *H. hircinum* was not previously reported and is 0.76 pg DNA for *H. maculatum* and for *H. umbellatum*, whereas *H. hircinum* has 1.00 pg DNA.

This study demonstrated that both ISSR and SRAP markers were highly polymorphic in *Hypericum*, showing the prevalence of a wide range of diversity among the studied accessions. The relative performance of ISSR and SRAP

markers was quite close, indicating that these markers are suitable for the determination of genetic diversity with high resolution among the *Hypericum* genotypes tested. Overall, marker analysis ensures information for potential applications of the SRAP and ISSR marker systems in molecular breeding of *Hypericum* species. Complementary analysis of ploidy level and molecular markers of different accessions of *Hypericum* species could provide information for the selection of valuable accessions producing high level of natural compounds useful for biotechnological applications.

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