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Phytochemical analysis and genetic characterization of six *Hypericum* species from Serbia

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Abstract

The secondary metabolite contents and genetic profiles of six *Hypericum* species (*H. barbatum* Jacq., *H. hirsutum* L., *H. linarioides* Bosse, *H. maculatum* Crantz, *H. rumeliacum* Boiss. and *H. tetrapterum* Fries), collected from different locations in Serbia, have been analyzed. Methanol extracts of the aerial parts of the plants were obtained by accelerated solvent extraction (ASE) at 40 °C and 100 bar, and analyzed for five pharmacologically important standard constituents (hyperoside, quercitrin, pseudohypericin, hyperforin and hypericin) by LC–MS/MS. The highest content of hypericin and pseudohypericin was observed in the *H. barbatum* extract, while the highest content of hyperforin and quercitrin was found in the *H. tetrapterum* extract and the highest content of hyperoside in the *H. maculatum* extract. A literature survey shows that the above six *Hypericum* species, with the exception of *H. maculatum*, have not been previously genetically profiled. In order to correlate the chemical constituents of the species under investigation with their genetic factors, genetic profiling of these species was undertaken using the random amplification of polymorphic DNA (RAPD) and single sequence repeat (SSR) profiles of the above selected plants. Among the 52 random primers used for the initial screening, only 10 yielded polymorphic RAPD profiles. A total of 111 polymorphic markers were generated using these primers. The SSR analysis shows that 8 out of the 10 primers used were polymorphic. The correlation among the species under investigation using the two genetic markers was performed using Jaccuard's coefficients of similarity and a high correlation ($r = 0.99$) was obtained. The main conclusion from the above data is that there exists a stronger correlation for secondary metabolite contents with RAPD data than with SSR data among the six *Hypericum* species from Serbia.

Keywords: *Hypericum barbatum*; *Hypericum hirsutum*; *Hypericum linarioides*; *Hypericum maculatum*; *Hypericum rumeliacum*; *Hypericum tetrapterum*; ASE; LC–MS/MS; RAPD; SSR

1. Introduction

Plant species of the genus *Hypericum* are well known for their use in traditional medicine due to their therapeutic efficacy. One of the most important and commercially recognized species of the genus is *H. perforatum* L. (St. John's wort), which has been used in herbal medicine, externally for the treatment of skin wounds, eczema and burns, and internally for disorders of the central nervous system, the alimentary tract and other ailments (Bombardelli and Morazzoni, 1995; Barnes et al., 2001). The main constituents of the *Hypericum* species are: naphthodianthrones, primarily represented by hypericin and pseudohypericin; flavonoids, e.g., hyperoside, rutin or quercitrin; and phloroglucinol derivatives, e.g., hyperforin and adhyperforin (Nahrstedt and Butterweck, 1997).

The objective of the present study was to determine and compare the secondary metabolite contents and genetic

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profiling of six wild-grown *Hypericum* species (*H. barbatum*, *H. hirsutum*, *H. linarioides*, *H. maculatum*, *H. rumeliacum* and *H. tetrapterum*) from Serbia. Initial data on the phytochemical analyses of six investigated species have been reported (Brantner et al., 1994; Kartnig et al., 1996; Kitanov, 2001; Maffi et al., 2001; Maggi et al., 2004; Umek et al., 1999). Recently, a detailed study on the secondary metabolite contents of nine *Hypericum* species, collected from southeastern Serbia in 2003, was performed, showing varying chemical contents on the basis of eco-geographical location and species (Smelcerovic and Spiteller, 2005). The earlier data showed that the content of hypericins from *H. perforatum*, obtained by accelerated solvent extraction (ASE), was higher than that obtained by Soxhlet extraction or ultrasound maceration. The optimum conditions for ASE extraction were 40 °C and 100 bar, using methanol as extraction solvent (Morf et al., 1998), and in the present investigation the extracts were obtained under the same conditions. The compositions of the extracts were analyzed using a modification of the LC–MS/MS method reported by Ganzera et al. (2002).

Characterization of plant germplasm using molecular techniques plays an increasingly important role in the management and utilization of plant genetic resources, but each molecular technique has limitations in the screening of large numbers of accessions held in seed gene banks worldwide. Therefore, more than one molecular markers should be used to come to a logical conclusion. The development of isozyme and/or DNA databases is a prerequisite to variety/species identification and protection. The genetic fingerprint provides a detailed description of both the raw material and the products for plant breeding and production. There are many polymorphic DNA markers available for identification and protection of genotypes. The time required for these types of analysis (Ribotyping, RAPD fingerprinting, AFLP fingerprinting, Microsatellite markers, etc.) depends primarily upon the type of assay, which must be agreed upon on a case-by-case basis. Literature survey shows that a PCR-based DNA amplification method, representing the nuclear ribosomal gene sequences of the internal transcribed spacer (ITS) region, had been applied to genetically distinguish fifty *Hypericum* taxa native to the Old and New Worlds (Crockett et al., 2004). Earlier, RAPD fingerprint analysis had been used to study the polymorphism, identity, or to check adulterants in populations and also to correlate this data with the mode of reproduction (by apomixis versus self-pollination, haploid parthenogenesis, or cross-fertilization) in *H. perforatum* (Arnholdt-Schmitt, 2000, 2002). However, reproduction in Australian populations of *H. perforatum* was studied (Mayo and Langridge, 2003) using restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP). Methylation of ribosomal DNA was used to distinguish between diploid and tetraploid taxa belonging to *H. perforatum* (using *Msp*I and *Hpa*II and 11 other methylation-sensitive restriction enzymes) (Haluskova et al., 2003). The literature survey shows that the six *Hypericum* species under investigation here have not been previously genetically profiled, with the exception of *H. maculatum* (Crockett et al., 2004).

In this paper, we report an attempt to correlate the data obtained from botanical (Robson, 1977), chemical and genetic profiles of six species of *Hypericum*. Such a correlation would be useful for plant breeding, quality control, intellectual property rights (IPR) and, eventually, for pharmacological studies.

2. Results and discussion

A typical LC–MS/MS chromatogram of the *Hypericum* extraction is presented in Fig. 1. Retention times, precursor and product ions, as well as collision energies for the standard compounds, are shown in Table 1. The highest content of hypericin and pseudohypericin was observed in the *H. barbatum* extract, while the highest content of hyperforin and quercitrin was found in the *H. tetrapterum* extract and the highest content of hyperoside in the *H. maculatum* extract (Table 2). Hypericin was found in all species, which is in agreement with our previous study (Smelcerovic and Spiteller, 2005) and the study of hypericins in some *Hypericum* species planted in Bulgaria (Kitanov, 2001). Pseudohypericin was not observed in *H. hirsutum* and *H. linarioides* (section *Taeniocarpium*). All the species studied contained hyperforin, as noted in our previous study (Smelcerovic and Spiteller, 2005), but this result contrasted with earlier phytochemical studies of *Hypericum* species (Umek et al., 1999; Maggi et al., 2004). In all studied species, with the exception of *H. tetrapterum*, hyperforin was found for the first time (Smelcerovic and Spiteller, 2005). The content of hyperforin in *H. perforatum* (Smelcerovic and Spiteller, 2005; Umek et al., 1999) was significantly higher than that found in the studied species.

There were significant differences in the active compound contents of samples collected from the same location (Table 2; locality Suva planina, samples of *H. barbatum*, *H. hirsutum*, *H. linarioides* and *H. maculatum*; locality Rudina planina, samples of *H. rumeliacum* and *H. tetrapterum*), which suggests that genetic factors may play a role in determining the active compound contents.

Among the 52 random primers used for the initial screening, only 10 yielded optimum RAPD profiles (Table 3). A total of 111 polymorphic markers were generated using these primers (Fig. 2(a)). The number of bands ranged from 4 to 10 per species and the amplified products varied in size between 50 and 2500 bp. The average number of bands per primer varied from 15 (PL-159) to 7 (PL-145) with an average of 11. RAPD data from the 10 primers were used for cluster analysis. The primers with G + C content of approximately 60% resulted in better polymorphism. The average proportion of polymorphic markers across primers was 93%, ranging between 81% (PL-146) and 100% (PL-126, PL-143, PL-159 and PL-160) (Table

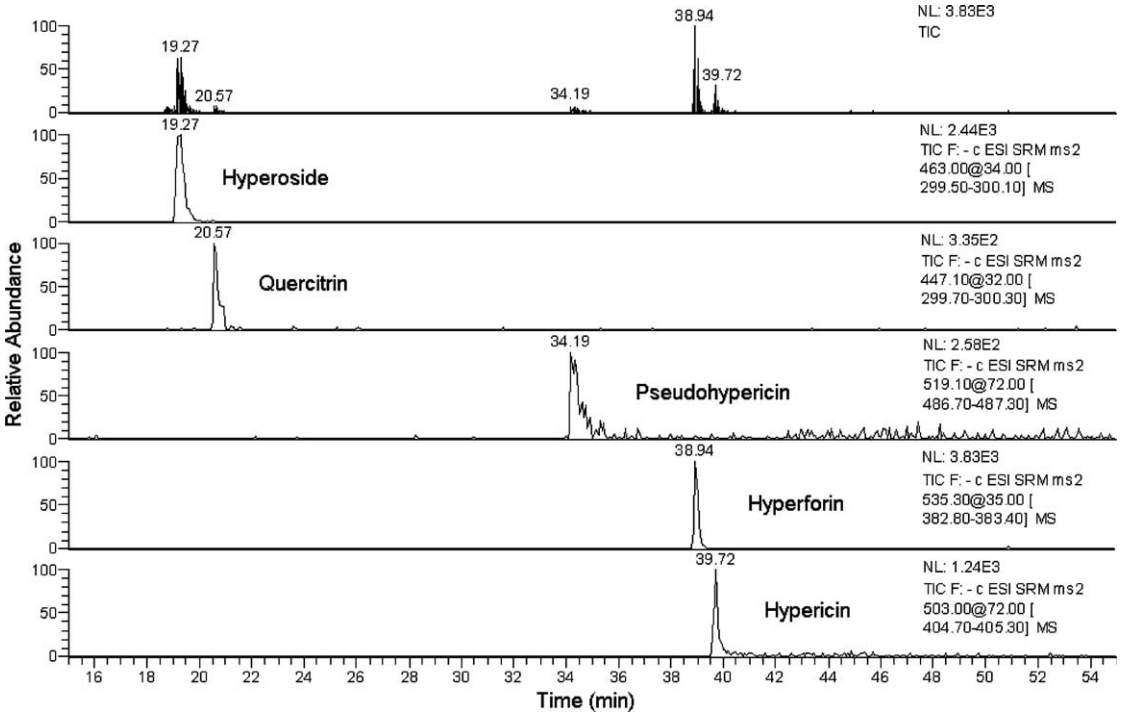

Fig. 1. LC–MS/MS chromatogram of *Hypericum barbatum* Jacq. extract.

Table 1
Retention times, precursor and product ions and collision energy for the standard compounds

Compound	Retention time	Precursor ion (*m*/*z*) $[M-H]^-$	Product ion (*m*/*z*)	Collision energy (eV)
Hyperoside	19.3	463	300	34.0
Quercitrin	20.6	447	300	32.0
Pseudohypericin	34.2	519	487	72.0
Hyperforin	38.9	535	383	35.0
Hypericin	39.7	503	405	72.0

3). The large number of exclusive markers account for a substantial portion of the genetic diversity, as illustrated by the mean Shannon index per primer of 3.53, with values ranging from 1.70 (PL-145) to 4.57 (PL-159) (Table 3). In case of RAPD, PL-159 generated a robust banding pattern and was found suitable for the species diversions alone.

Eight out of the 10 SSR primers (Table 4) exhibited polymorphism among the six *Hypericum* species under study. A total of 50 bands were generated by these primers. The number of bands per primer ranged from four (PL 575 and PL 576) to nine (PL 589 and PL 590). The average percentage of polymorphic markers across the eight primers was 90.6%. All primers except PL 575 and PL 576 generated the highest level of polymorphism (Table 4) as they amplified maximum number of polymorphic bands. The average Shannon index obtained with eight SSR primers was 1.72. The highest banding pattern was obtained with PL 589 and PL 590 (Table 4). The percent polymorphism generated by them was 100%. The results obtained from SSR analysis (Fig. 2(b)) show the efficiency of such markers in exploring the genetic diversity among *Hypericum* species. Cluster analysis (unweighted pair group method with arithmetic averages, UPGMA) was used to generate two independent dendrograms for the two types of markers. The correlation between the two markers was calculated using Jaccuard's coefficients of similarity. A significant similarity ($r = 0.99$) was obtained.

While the dendrograms generated from the RAPD and SSR data (Fig. 3) are in partial agreement, in terms of hypothesized phylogenetic relationship, differences are also shown. In both dendrograms, *H. linarioides* alone forms a separate entity. This corroborates the chemical data, because this species is the only one of plant species under study in which quercitrin is absent. The correlation of active compound contents with the RAPD and SSR marker data conforms with the data based on infrageneric parameters obtained by Robson (1977). The highest contents of hypericin and pseudohypericin were observed in *H. barbatum* and *H. rumeliacum* and the content of hyperforin in these species was the same. According to infrageneric classification (Robson, 1977), these species belong to the same section (*Drosocarpium*) and in the RAPD dendrogram (Fig. 3(a)) these two species form a subcluster. The

Table 2
Relevant data on the studied *Hypericum* species from Serbia and their secondary metabolite contents

Section	Plant species	Voucher number (HMD)	Collection period	Locality	Hyperoside content (mg/g)	Quercitrin content (mg/g)	Pseudohypericin content (mg/g)	Hyperforin content (mg/g)	Hypericin content (mg/g)
Hypericum	*H. maculatum* Crantz	726	August 2004	Suva planina, East Serbia	6.61	0.08	0.04	0.05	0.03
	H. tetrapterum Fries	731	July 2004	Rudina planina, South east Serbia	6.01	0.80	0.10	0.11	0.09
Drosocarpium	*H. barbatum* Jacq.	723	August 2004	Suva planina, East Serbia	1.55	0.19	0.43	0.07	0.30
Spach	*H. rumeliacum* Boiss.	730	July 2004	Rudina planina, South east Serbia	0.29	0.43	0.18	0.07	0.18
Taeniocarpium	*H. hirsutum* L.	724	August 2004	Suva planina, East Serbia	0.94	0.32	–	0.06	0.04
Jaub. and Spach	*H. linarioides* Bosse	725	August 2004	Suva planina, East Serbia	2.71	–	–	0.02	0.02

species *H. maculatum* and *H. tetrapterum* belong to the section *Hypericum*; they had the significantly higher content of hyperoside and this is in agreement with the RAPD data (Fig. 3(a)), as it forms a single subcluster of the two species.

The main conclusion from above data is that there exists a stronger correlation between the secondary metabolite contents and RAPD data among the six *Hypericum* species from Serbia than between secondary metabolite contents and the SSR data. As the SSR data represent repeat sequences, it is possible that the species have deviated more from one another in the course of their evolution with respect to the repeat sequences, than the general sequences of the genome and those encoding for the chemical constituents. Our data indicate that RAPD maker data would be very useful for the plant breeders and for the purposes of identification and possibly quality control of the species under study.

3. Experimental

3.1. Materials

Hyperoside was purchased from Merck (Germany), quercitrin from Sigma (Germany), pseudohypericin from Calbiochem (Germany), hyperforin from Cayman Chemical (USA) and hypericin from Biomol GmbH (Germany). Ammonium acetate and glacial acetic acid, as well as the solvents acetonitrile (Merck, Germany), methanol (Merck, Germany), and ultra-pure water (Millipore, Germany), were HPLC grade.

Table 2 contains data concerning the identity of the *Hypericum* species under study, voucher numbers of the deposited herbarium specimens (Herbarium Moesicum Doljevac, Serbia and Montenegro), site and date of collection, as well as their taxonomic placement within sections of the genus *Hypericum* (Robson, 1977). All the plant species were collected at bloom stage. Fresh plant materials were used for the DNA fingerprinting. The plant materials used for phytochemical analysis were dried at room temperature and then ground.

3.2. Preparation of plant extracts

ASE was performed using a Dionex (Sunnyvale, CA) ASE 200 accelerated solvent extractor equipped with 11 ml stainless steel cells and 60 ml collection vials. All extractions were performed using 0.5 g dry plant material. ASE conditions were as follows: extraction solvent, methanol; temperature, 40 °C; pressure, 100 bar; 4 cycles with static extraction time of 5 min; flush volume, 60%; final solvent volume, 30 ml. The extracts were stored in the dark at 4 °C.

3.3. Determination of secondary metabolite contents

HPLC analysis of the extracts was performed using a Dionex HPLC system, equipped with a UVD 340s photo-

Table 3
Comparison of genetic diversity and dissimilarity coefficients among the six *Hypericum* species under study by RAPDs

Primer	Sequence	Percentage GC content	Total number of bands	Percentage polymorphism	Shannon index
PL-4	5′-GGACTGGAGT-3′	60	13	92	3.54
PL-51	5′-AGCGCCATTG-3′	60	13	92	3.54
PL-71	5′-AAAGCTGCGG-3′	60	12	91	3.27
PL-126	5′-GGGAATTCGG-3′	60	11	100	2.66
PL-143	5′-TCAGGGAGGT-3′	60	10	100	3.30
PL-145	5′-GACGGATCAG-3′	60	7	85	1.70
PL-146	5′-AAGACCCCTC-3′	60	11	81	2.20
PL-159	5′-ACGGCGTATG-3′	60	15	100	4.57
PL-160	5′-AACGGTGACC-3′	60	10	100	3.28
PL-175	5′-CCAGATGCAC-3′	60	9	88	2.47

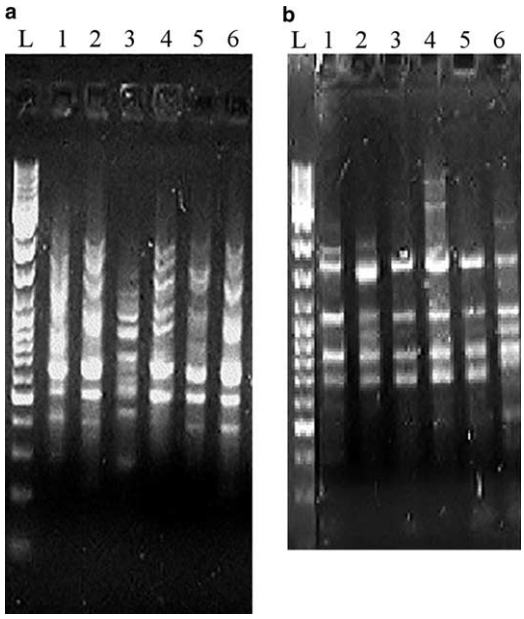

Fig. 2. RAPD (a) and SSR (b) profiles of the six species under study (lane L 100 bp ladder; lane 1 *Hypericum tetrapterum* Fries; lane 2 *Hypericum maculatum* Crantz; lane 3 *Hypericum linarioides* Bosse; lane 4 *Hypericum rumeliacum* Boiss.; lane 5 *Hypericum hirsutum* L.; lane 6 *Hypericum barbatum* Jacq.).

diode array detector and a Gina 50 autosampler. Separations were performed on a Luna 3u C18 100 Å column (150 × 2 mm, 3 μm particle size) from Phenomenex (Torrance, CA). The mobile phase consisted of 10 mM ammonium acetate buffer adjusted to pH 5.0 with glacial acetic acid (A) and a 9:1 mixture of acetonitrile and methanol (B) (Ganzera et al., 2002). Gradient elution was performed using the following solvent gradient: from 87A/13B in 10 min to 83A/17B, then in 25 min to 10A/90B and in 5 min to 100B; each run was followed by an equilibration period of 10 min. The eluent flow rate was 0.25 ml/min and the injection volume was 10 μl. All separations were performed at 24 °C.

Mass spectra were obtained using a TSQ 7000 mass spectrometer (Thermo Finnigan, Bremen, Germany) equipped with an ESI source operating in negative mode. For ESI, the best conditions were found to be: ionization voltage at 4 kV; transfer capillary temperature at 220 °C; tube lens and skimmer at 70 V. Nitrogen was employed as both the drying and nebuliser gas. The ionization current was fixed at 5 kV and the detector voltage at 1200 V for MS and 1500 V for MS/MS. The calibration curve was constructed by dilution of standards with methanol to give the desired concentrations. The concentrations of

Table 4
Comparison of genetic diversity and dissimilarity coefficients among the six *Hypericum* species under study by using eight sets of SSR primers

Primer	Sequence	Total number of bands	Percentage polymorphism	Shannon index
PL 553 and PL 554	5′-TGCACGGGGAAGAGGAGAGA-3′ 3′-AACCGAAGCGCAAGAACCCA-5′	5	100	1.72
PL 561 and PL 562	5′-CGCCGCCGTACTGCTCCATC-3′ 3′-GCGGAGGAGACCTGCGGGT-5′	8	100	1.57
PL 569 and PL 570	5′-CTCGCCGTCGAATCCGCCAT-3′ 3′-CACTCTCCTCTCCTGGCCCG-5′	6	100	1.40
PL 575 and PL 576	5′-GTTTCTCACGTCTCTCTCGCTG-3′ 3′-TCCTCCTCCTACGGCTTCTC-5′	4	25	0.43
PL 581 and PL 582	5′-TTTATCCGCGTCCCTAGCTT-3′ 3′-GCCGCCGGGGTCACAGGTCA-5′	6	100	1.83
PL 589 and PL 590	5′-ATATATCCAGCCAGCCGAT-3′ 3′-GGGCCGTGCCGTGCCTCACC-5′	9	100	2.80
PL 591 and PL 592	5′-CTCCTCGATCTTCCTCTACT-3′ 3′-TCGACCCCATCACAAATCCA-5′	5	100	1.28
PL 595 and PL 596	5′-GGGATGATCATCTCCGATGC-3′ 3′-CCCTTCTCCCACTCTTCTCC-5′	7	100	2.75

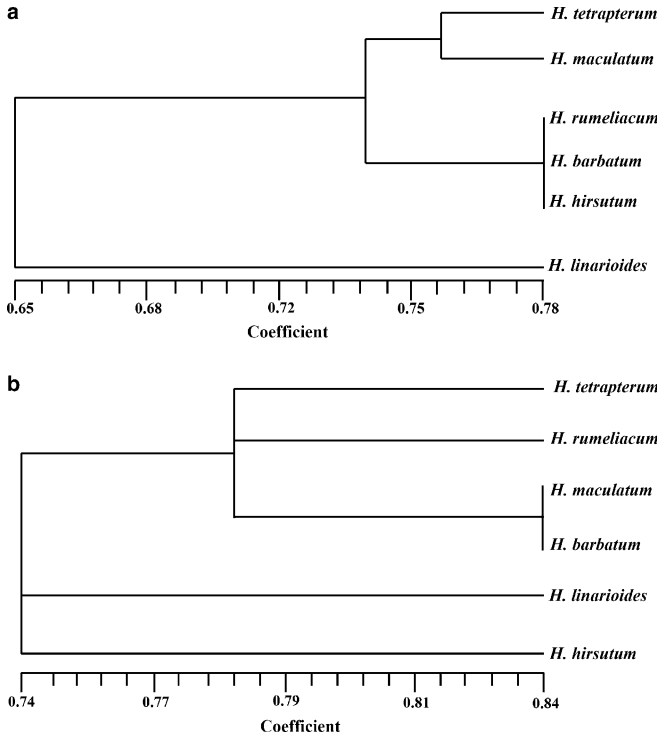

Fig. 3. Dendogram obtained via RAPD (a) and SSR (b) of the species under study.

the standard solutions were: 1, 2.5, 5, 10 and 20 μg/ml for quercitrin; 1, 2.5, 5 and 20 μg/ml for hyperosid; 0.5, 1, 2.5, 5 and 10 μg/ml for pseudohypericin; 0.1, 0.5, 1, 2.5, 5, 10 and 20 μg/ml for hyperforin; 0.5, 1, 2.5 and 10 μg/ml for hypericin. Standard solutions were stored in the dark at 4 °C. All procedures were carried out under light protection. Within the range of concentrations injected the detector response (peak area) was linear. For quantitative analysis, the peak area of each selected component was measured using Xcalibur 1.3 software (Thermo Finigan).

3.4. DNA extraction

Total genomic DNA was isolated from fresh leaves following the method of Ahmad et al. (2004). The leaf tissue was washed in running tap water followed by doubly distilled water to remove dust and then air dried before use for DNA isolation. Estimation of DNA quantity and quality was performed by spectophotometric analysis (Bio Photometer, Eppendorf, Germany) and agarose gel (0.8%) electrophoresis.

3.5. RAPD analysis

In total, 52 decamer RAPD primers (Operon, USA) were screened and, finally, 10 primers (Table 3) were selected, as these generated reproducible polymorphism in the species under investigation. PCR was performed in a volume of 20 μl containing 2 μl of 10× *Taq* DNA polymerase buffer, 2 μl of 2.25 mM $MgCl_2$, 2 μl of 0.2 mM of dNTP mix, 1 μl of 0.36 μM of each primer (Bio Basic Inc., Canada), 2 μl containing 0.4 ng genomic DNA, and 1 unit of *Taq* DNA polymerase (Bio Basic Inc., Canada). DNA amplification was performed in a Master Cycler Gradient (Eppendorf, Germany). After initial incubation for 3 min at 95 °C, the samples for enzymatic amplification were subjected to 40 cycles of the following PCR program: 30 s at 94 °C, 1 min at 35 °C and 1 min at 72 °C. The final extension at 72 °C for 10 min ended the program. After amplification, the reaction products were fractionated by electrophoresis in 1.5% agarose gels in 1× TAE buffer at 5 V/cm; the gels were stained with ethidium bromide and photographed under UV light using a gel documentation system (Ultra Cam, Germany). Gene Rular™ 100 bp plus (Fermantas, Germany) was used as the molecular standard in all gel electrophoresis. All PCR results were tested for reproducibility at least three times. Bands that did not show fidelity were eliminated.

3.6. SSR analysis

The SSR were amplified using the primers designed by Miyao et al. (1996). PCR was performed in a volume of 50 μl containing 5 μl of 10× *Taq* DNA polymerase buffer, 2 μl of 2.25 mM $MgCl_2$, 2 μl of 0.2 mM of dNTP mix, 0.75 μl of each primer (0.05 μM), 2 μl (6 ng) of template DNA and 1 unit of *Taq* DNA polymerase (Bio Basic Inc., Canada). The reaction mixtures were incubated in a Master Cycler Gradient (Eppendorf, Germany) with the following PCR program: 95 °C for 3 min, 94 °C for 30 s, 45 °C for 1 min, 72 °C for 1.30 min. The program was repeated twice before the final extension at 72 °C for 10 min and linked to a program consisting of 30 cycles of 95 °C for 30 s, 58 °C for 1 min, 72 °C for 1.30 min. The reactions were stopped with a final extension for 10 min at 72 °C. Amplified products were separated on 1.5% agarose gels run at 5 V/cm in 1× TAE buffer; the gels were stained with ethidium bromide and photographed under UV light using a gel documentation system (Ultra Cam, Germany). Gene Rular™ 100 bp plus (Fermantas, Germany) was used as the molecular standard in all gel electrophoresis. All the PCR and restriction digestion results were tested for reproducibility.

3.7. Data analysis

For RAPD and SSR polymorphic, reproducible amplification/restriction products were scored as present (1) or absent (0). Genetic diversity was estimated by the Shannon index (Lewontin, 1972):

$$H = -\sum_{i=1}^{k} pi \ln pi,$$

where K is the number of bands produced with the respective primer/restriction enzyme and pi is the frequency of the ith fragment.

To further investigate phenetic relationships among accessions, the binary matrix was used to cluster individuals using procedure of NTSYS-pc1.8 (Rohlf, 1993), which uses the UPGMA. The estimates of pair wise genetic distance between six *Hypericum* species were based on Jaccuard's similarity coefficient.

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