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Adoption of RAPD-related methodology for analyses of *Hypericum maculatum* Crantz growing wild in Lithuania

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Hypericum genus plants are widespread all over Europe and Asia and they are one of the most important world plants used for medicinal purposes. In Lithuania insufficient attention is paid to the second according to the abundance species of *Hypericum* genus – *H. Maculatum* L. Till now research of this species was mainly concentrated on morphology and some chemical parameters. The present study aimed at selection of the conditions for random amplification of polymorphic DNA extracted from *H. maculatum* growing wild in Lithuania. NucleoSpin Plant II (Macherey-Nagel, Germany) as the most efficient kit was selected for DNA extraction from *H. maculatum*. For polymerase chain reaction (PCR) 14 nucleotide primers (Biomers.net, Germany) were used and OP-A1, OP-A4, OP-A9, OP-B12, OP-B19, OP-C6a, OP-C7a, OP-C11, OP-C19, OP-D20, OP-Q2 were selected for further studies of genetic diversity of populations of *H. maculatum* (OP-B13, OP-D19a, OP-Q11 were rejected). DNA amplification program with 40 cycles was chosen from 35, 40 and 45 cycles tested. Among two different composition mixes used for PCR, more efficient was the one consisting of: 0.05 U/µl *Taq* DNA polymerase; 1 × *Taq* buffer; 1.5 nM $MgCl_2$; 0.2 mM dNTP; 2 µl primer (10 pmol/µl) and 14.55 µl deionised water (Fermentas, Lithuania). For preparation of above mentioned mix 3 different types of *Taq* buffer were tested: 1 – *Taq* buffer with KCl; 2 – *Taq* buffer with $(NH_4)_2SO_4$; 3 – *Taq* buffer without detergent. The best results were achieved with the first buffer.

Key words: imperforate St John's wort, DNA markers, genetic diversity, populations, *Hypericaceae*

INTRODUCTION

The genus *Hypericum* is common all over Europe and Asia. In Lithuania two species are widely spread: common St John's wort (*Hypericum perforatum* L.) and imperforate St John's wort (*Hypericum maculatum* Crantz) [19]. Over thirty different cases of application of *H. perforatum* are described [23] and it is one of the most widely investigated herbal plants [24]. The main secondary constituents of *H. perforatum* are naphtodianthrones, phloroglucinols, flavonoids and phenolic acids [9]. The most important components of essential oils of *H. perforatum* are β-Caryophyllene, caryophyllene and germacrene D [8]. Similar ones are characteristic for *H. maculatum* [3]. Among all molecular markers, DNA markers are the most suitable and ubiquitous to various living organisms [2, 22]. During several decades there has been a revolution in understanding of technologies and of the scale of application of molecular genetics approaches to physiological and ecological problems in the plant sciences. The reasons for wishing to map a gene are as diverse as the fields of biology that have been affected by molecular genetics. All types of DNA variation are potentially useful for the mapping of molecular markers, and with the exception of simple sense base substitutions with no phenotypic outcome, may also result in detectable variation in trait expression [1]. The most popular method for plant analysis is Random Amplified Polymorphic DNA (RAPD) [5, 16,

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12, 13]. Wide scope research has been done concerning *H. perforatum* and *H. maculatum* growing wild in Lithuania. Data collected investigating Lithuanian populations of *Hypericum* mainly concern morphology and chemistry [25, 20, 21, 15, 17]. In other countries the genetic diversity of *Hypericum* is precisely characterized and species specific markers of *H. perforatum* have been identified [18, 6], chloroplast DNA variability among some *Hypericum* species has also been documented [7]. Reproductive biology in ecotypes of the facultative apomicts *H. perforatum* L. is analyzed [27] and cytogenetic variability of regenerants is evaluated [14]. Variations of somaclonal *Hypericum* plants according to DNA have been described [4]. Despite the fact that plant genetic polymorphism is a subject of wide scope investigations in Lithuania, till now there are no data concerning genetic variability of Lithuanian populations of *H. maculatum*. The first step for genetic polymorphism studies is the elaboration of precise conditions for DNA analyses adapted to some species. The present study aimed at selection of the conditions for random amplification of polymorphic DNA extracted from *H. maculatum* growing wild in Lithuania.

MATERIALS AND METHODS

Plant material. The plant material was collected from various locations of Lithuania in June–July, 2009. Leaves from healthy plants were collected and frozen.

DNA extraction. The amount of leaf tissue used for DNA extraction ranged from 0.03 to 0.3 g. Genomic DNA was isolated using two methods: Genomic DNA purification kit (Fermentas, Lithuania) according to the protocol of producer with some modifications [10, 28] and NucleoSpin Plant II (Macherey-Nagel, Germany) according to the protocol of producer. The concentration of DNA samples was determined using spectrophotometer (Eppendorf BioPhotometer, Germany). The concentration of DNA samples was determined by optical density reading at 260 nm (1 OD = 50 μg/ml) and their purity calculated by the OD_{260}/ OD_{280} ratio and the OD_{210}/ OD_{310} pattern [27]. Further quality of DNA samples was assessed by electrophoresis on 1.5% agarose gel [11].

RAPD analysis. Fourteen oligonucleotide primers of 10 nt length (Biomers.net, Germany) were used (Table 1) according to investigations of genetic diversity and reproductive biology in ecotypes of the facultative apomicts *H. perforatum* [14].

Two different PCR mixes were used for RAPD-PCR reaction. The first mix of PCR was prepared as follows: one sample in a volume of 25 μl contained 12.5 μl 2 × PCR Master Mix (0.05 U/μl *Taq* DNA polymerase; *Taq* buffer; 4 mM $MgCl_2$; 0.4 mM each dNTP; Fermentas, Lithuania), 2 μl of each primer (0.8 pmol/μl), 6.5 deionised water and 4 μl DNA. The second PCR mix was prepared in a volume of 25 μl consisting of: 0.05 U/μl *Taq* DNA polymerase; 1 × *Taq* buffer; 1.5 nM $MgCl_2$; 0.2 mM dNTP; 2 μl primer (10 pmol/μl); 14.55 μl deionised water (Fermentas, Lithuania) and 4 μl DNA. For the second PCR mix three different types of the buffer were tested: 1) buffer with KCl (Cat. #B38); 2) buffer with $(NH_4)_2SO_4$ (Cat. #B33); 3) buffer without detergent (Cat. #B55). As a negative control a sample without DNA and with deionised water was used.

Table 1. **List of the RAPD primers**

Primer	Sequence (5′–3′)
OP-A1	CAGGCCCTTC
OP-A4	AATCGGGCTG
OP-A9	GGGTAACGCC
OP-B12	CCTTGACGCA
OP-B13	TTCCCCCGCT
OP-B19	ACCCCCGAAG
OP-C6a	GAACGGACTC
OP-C7a	GTCCCGACGA
OP-C11	AAAGCTGCGG
OP-C19	GTTGCCAGCC
OP-D19a	CTGGGGACTT
OP-D20	ACCCGGTCAC
OP-Q2	TCTGTCGGTC
OP-Q11	TCTCCGCAAC

DNA amplification was performed in Mastercycler gradient (Eppendorf, Germany) according to the following program: first denaturation for 2 min at 94 °C; 35, 40 or 45 cycles which includes denaturation for 30 s at 94 °C, primers annealing for 35 s at 32 °C or 34 °C (depending on primer melting temperature or lower), extension for 1 min at 72 °C and the final extension for 2 min at 72 °C.

The reaction products were fractionated by electrophoresis in 1.5% agarose gel with ethidium bromide and the photographs of gels in the UV light were taken using Herolab transliuminator, Germany.

The lengths of fragments were estimated according to the standard molecular marker of 100 bp (Gene ruler DNA Ladder, Fermentas, Lithuania). Fragments with the same mobility were evaluated as identical DNA fragments.

RESULTS AND DISCUSSION

The selection of DNA extraction method. Genomic DNA was extracted from 150 mg *H. maculatum* leaves, collected from 4 different locations. Genomic DNA purification kit (Fermentas, Lithuania) was used. The quantity and quality of DNA was estimated by spectrophotometer (Table 2) and later on by electrophoresis on 1.5% gels.

The purity of extracted DNA samples was very high but the concentration was low and electrophoresis analyses did not give visible fragments.

Table 2. **DNA concentration and purity using DNA extraction kit (Fermentas, Lithuania)**

Plant samples from different locations	DNA concentration (ng/µl)	DNA purity (260 nm/280 nm)	DNA purity (320 nm)
1	6.84	1.7	0.005
2	7.99	1.69	0.006
3	7.82	1.74	0.002
4	4.18	1.56	0.006

Genomic DNA was repeatedly extracted with the same DNA extraction kit homogenizing larger amount of plant material up to 300 mg. Again, electrophoresis analyses did not give visible fragments. After such experiment, genomic DNA purification kit (Fermentas, Lithuania) was not used for further *Hypericum* DNA extraction.

For the subsequent experiments, DNA was extracted using NucleoSpin Plant II DNA extraction kit (Macherey-Nagel, Germany). Approximately 30 mg of frozen leaves were used according to the company's recommendations. DNA was extracted from 2 different plants collected in each of 10 locations. In total, twenty samples were tested. The quality of DNA samples was assessed by the above described methods. Electrophoresis of DNA gave clear and visible fragments. The concentration of DNA estimated by spectrophotometer ranged from 5.06 to 26.9 ng/µl, average DNA concentration was 13.9 ng/µl.

Optimization of conditions for DNA amplification. Firstly, we tried to make PGR reaction in accordance with the previous works performed at our laboratory [10, 28] using 2 × PCR Master Mix (Fermentas, Lithuania) with 14 primers selected for genetic analysis of *H. perforatum* by other laboratories [14]. For about 50% of the analyzed individuals three primers (OP-A1, OP-A9 and OP-C11) out of fourteen gave quite informative fragments. Primer OP-A1 gave 7 amplified DNA fragments, 4 of them were polymorphic. The size of the amplified fragments ranged from 100 to 1 450 bp. Amplified fragment number for one individual ranged from 2 to 7. For separate individuals primer OP-A1 generated the highest number of fragments. Primer OP-A9 gave 6 amplified DNA fragments, 4 of them were polymorphic. The size of the amplified fragments ranged from 190 to 1 000 bp. For one individual the number of amplified fragments ranged from 1 to 6. Primer OP-A9 gave maximum 6 fragments for one individual. Primer OP-C11 gave 5 amplified DNA fragments, 3 of them were polymorphic. The size of the amplified fragments ranged from 340 to 1 200 bp. For one individual the number of amplified fragments ranged from 3 to 4. Primer OP-C11 gave maximum 4 fragments for one individual (Table 3). 2 × PCR Master Mix (Fermentas, Lithuania) appeared not applicable for other 9 primers.

Recently we have chosen to make PCR mix with different concentrations of components. We tested three mixes with different buffers of *Taq* reaction: *Taq* buffer with KCl, *Taq* buffer with $(NH_4)_2SO_4$ and *Taq* buffer without detergent. For all tested samples of plant material the best output was achieved using *Taq* buffer enriched with KCl and without $MgCl_2$ (Figure).

Table 3. **DNA amplification results with 3 primers using 2 × PCR Master Mix (Fermentas, Lithuania)**

Primer	Amplified DNA fragment number	Length of the fragments of amplified DNA	Number of polymorphic fragments of DNA	Number of amplified fragments for one individual
OP-A1	7	100–1 450	4	2–7
OP-A9	6	190–1 000	4	1–6
OP-C11	5	340–1 200	3	3–4

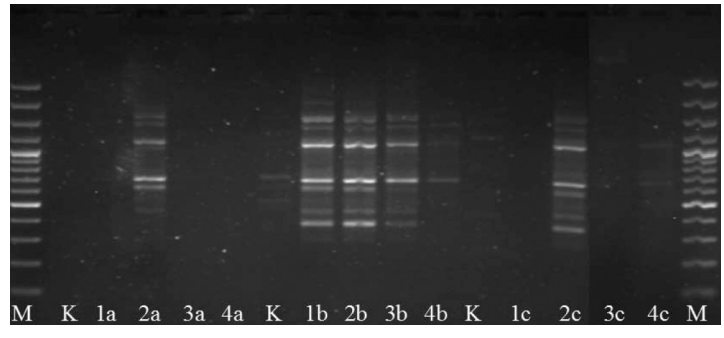

Figure. RAPD amplification results for *Hypericum maculatum* with primer OP-A1 and different *Taq* buffers (1, 2, 3, 4 different individuals; a – *Taq* buffer with $(NH_4)_2SO_4$; b – *Taq* buffer with KCl; c – *Taq* buffer without detergent; M –100 bp DNA size marker)

Attempts were made to select a program of DNA amplification. Firstly, we have tested the effect of different number of denaturation cycles to obtain clearly visible fragments of DNA. Better results were achieved when denaturation cycles were reduced from 45 to 40. 35 cycles did not provide DNA amplification products for the following primers: OP-C6a, OP-C11, OP-D19a, OP-Q2, OP-Q11. For those primers 40 cycles were more suitable. For other primers: OP-A1, OP-A9, OP-B19, OP-C7a, OP-C19, OP-D20, both 40 and 35 cycles gave scorable fragments, although fragments obtained in 40 cycles were of better appearance.

For all examined primers several annealing temperatures were tested. For all informative primers the best annealing temperature was the same as the melting temperature of the primer (Table 4). Visible fragments were not obtained reducing temperature by 2 °C or 5 °C.

According to our study, 11 out of 14 analyzed primers could be used for the studies of *H. maculatum* genomic DNA (Table 5).

We found that primer OP-A1 gave 8 amplified DNA fragments, 5 of them were polymorphic. The size of the amplified fragments ranged from 300 to 1 500 bp. Amplified fragment number for one individual ranged from 4 to 8. Primer OP-A9 gave 5 amplified DNA fragments, 5 of them were polymorphic. The size of the amplified fragments ranged from 180 to 1 200 bp. Amplified fragment number for one individual ranged from 3 to 4. Primer OP-B19 gave 5 amplified DNA fragments, 4 of them were polymorphic. The size of the amplified fragments ranged from 200 to 2 000 bp. Amplified fragment number for one individual ranged from 2 to 3. Primer OP-C7a gave 5 amplified DNA fragments, 3 of them were polymorphic. The size of the amplified fragments ranged from 700 to 2 900 bp. Amplified fragment number for one individual ranged from 1 to 4. Primer OP-C19 gave 5 amplified DNA fragments, 3 of them were polymorphic. The size of the amplified fragments ranged from 500 to 1 400 bp. Amplified fragment number for one individual was 4. Primer OP-D20 gave 5 amplified DNA fragments, 5 of them were polymorphic. The size of the amplified fragments ranged from 300 to 2 000 bp. Amplified fragment number for one individual ranged from 2 to 4. Primer OP-A4 gave 7 amplified DNA fragments, 4 of them were polymorphic. The size of the amplified fragments ranged from 390 to 1 900 bp. Amplified fragment number for one individual ranged from 3 to 7. Primer OP-B12 gave 9 amplified DNA fragments, 6 of them were polymorphic. The size of the amplified fragments ranged from 450 to 2 200 bp. Amplified fragment number for one individual ranged from 4 to 8. Primer OP-C6a gave 5 amplified DNA fragments, 3 of them were polymorphic. The size of the amplified fragments ranged from 500 to 1 400 bp. Amplified fragment number for one individual was 4. Primer

Table 4. **Primer melting temperatures**

Primer	Primer melting temperature, °C	Primer	Primer melting temperature, °C
OP-A1	34	OP-A4	32
OP-A9	34	OP-B12	32
OP-B13	34	OP-C6a	32
OP-B19	34	OP-C11	32
OP-C7a	34	OP-D19a	32
OP-C19	34	OP-Q11	32
OP-D20	34	OP-Q2	32

Table 5. **RAPD analysis results of *Hypericum maculatum* using 14 primers**

Primer	Amplified DNA fragment number	Length of the fragments of amplified DNA	Number of polymorphic fragments of DNA	Number of amplified fragments for one individual
OP-A1	8	300–1 500	5	4–8
OP-A9	5	180–1 200	5	3–4
OP-B13	0	0	0	0
OP-B19	5	200–2 000	4	2–3
OP-C7a	5	700–2 900	3	1–4
OP-C19	5	500–1 400	3	4
OP-D20	5	300–2 000	5	2–4
OP-A4	7	390–1 900	4	3–7
OP-B12	9	450–2 200	6	4–8
OP-C6a	6	580–1 900	3	3–6
OP-C11	8	250–1 500	3	5–7
OP-D19a	0	0	0	0
OP-Q2	3	800–1 400	1	2–3
OP-Q11	0	0	0	0

OP-C11 gave 8 amplified DNA fragments, 3 of them were polymorphic. The size of the amplified fragments ranged from 250 to 1 500 bp. Amplified fragment number for one individual ranged from 5 to 7. Primer OP-Q2 gave 3 amplified DNA fragments, 1 of them was polymorphic. The size of the amplified fragments ranged from 800 to 1 400 bp. Amplified fragment number for one individual ranged from 2 to 3. Three primers OP-B13, OP-D19a and OP-Q 11 were not informative and did not give visible fragments for all DNR samples extracted with NucleoSpin Plant II kit.

CONCLUSIONS

For genomic DNA extraction from *H. maculatum* the NucleoSpin Plant II (Macherey-Nagel, Germany) was more efficient. Comparing two different tested PCR mixes, the mix with two times lower $MgCl_2$ and dNTP concentration was more efficient amplifying *H. maculatum* DNA. The best amplification results were achieved with 40 cycles of amplification. For all informative primers the best annealing temperature was the same as the primers melting temperature. Among 14 primers tested, 11 primers appeared to be informative for the studies of genetic diversity of populations of *H. maculatum*.

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ATSITIKTINAI PAGAUSINTOS POLIMORFINĖS DNR METODO SĄLYGŲ PARINKIMAS LIETUVOS KETURBRIAUNĖS JONAŽOLĖS POPULIACIJŲ TYRIMAMS

Santrauka

Jonažolės genties augalai, paplitę visoje Europoje ir Azijoje, yra vieni dažniausiai pasaulyje naudojamų vaistinių augalų. Antra pagal gausumą Lietuvoje šios genties rūšis – keturbriaunė jonažolė yra mažiau ištirta negu paprastoji jonažolė. Dažniausiai apsiribojama Lietuvos jonažolių morfologiniais ir kai kuriais cheminiais tyrimais. Šio darbo tikslas buvo pritaikyti atsitiktinai pagausintos polimorfinės DNR (APPD) metodo sąlygas keturbriaunės jonažolės Lietuvos populiacijų genetinės įvairovės tyrimams. Didžiausia keturbriaunės jonažolės DNR išeiga buvo gauta panaudojant NucleoSpin Plant II (Macherey-Nagel, Vokietija) rinkinį. Atliekant polimerazinę grandininę reakciją (PGR) išbandyta 14-a dešimties nukleotidų ilgio pradmenų (Biomers.net, Vokietija), iš jų 11 pradmenų (OP-A1, OP-A4, OP-A9, OP-B12, OP-B19, OP-C6a, OP-C7a, OP-C11, OP-C19, OP-D20, OP-Q11) buvo informatyvūs ir tinkami tolesniems keturbriaunės jonažolės genetinės įvairovės tyrimams, trys atmesti (OP-B13, OP-D19a, OP-Q11). Išbandžius įvairios sudėties PGR mišinius, DNR geriausiai pagausinta naudojant 10 × Taq buferį su KCl ir 15 mM $MgCl_2$ („Fermentas“, Lietuva). Iš 12–15 laipsnių intervale bandytų temperatūrų ir 15–40 ciklų intervale atlikto DNR pagausinimo atrinktos palankiausios DNR pagausinimo sąlygos: pagrindiniam gausinimui naudojama 40 ciklų, pradmenų prikibimui priklausomai nuo pradmens parenkama 32 arba 34 °C temperatūra.

Raktažodžiai: keturbriaunė jonažolė, *Hypericaceae*, atsitiktinai pagausinta polimorfinė DNR, genetinė įvairovė