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Development of microsatellite markers for *Rhodiola rosea*

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Summary: *Rhodiola rosea* L. is an important adaptogen medicinal plant. In this study two new microsatellite markers were developed. The assessment of the genetic diversity of *R. rosea* has recently started with molecular markers, but only a few species-specific microsatellite markers have been published so far. However the small number of markers allows only a limited insight into the genetic variability of the species therefore the aim of our work was to develop new microsatellite markers for *R. rosea* with a microsatellite enrichment library technique. Genomic DNA was cleaved with an endonuclease enzyme followed by adaptor ligation and PCR amplification. DNA fragments that contained microsatellites were first isolated using a biotin-streptavidin linkage based magnetic selection and then cloned into plasmids. Out of forty-three sequenced clones three contained microsatellites, in these cases primers were designed for the amplification of the microsatellite repeats. The newly developed primer pairs were tested on individuals from distant *R. rosea* populations and the variability of the amplified fragments was estimated by fragment-length analysis. The locus RhpB14a was found to be monomorphic while RhpB14b and RhpB13 were polymorphic. As a result of the present study, two novel variable microsatellite loci were identified in the genome of *R. rosea*.

Keywords: adaptogen, microsatellite marker, aromatic plant, population genetic, *Rhodiola rosea*

Introduction

Rhodiola rosea L. (roseroot or golden root) is a popular plant in traditional medicine because of its adaptogen properties. Adaptogenic herbs contain active substances that neutralize physical, chemical and biological stressors. *Rhodiola rosea* has a positive effect on cardiovascular functions as well as the ability to stimulate the central nervous system (*Kelly*, 2001). Because of the antioxidant and anti-psychotic effects of *R. rosea*, the extract is used to treat tiredness, depression, anemia, impotence, infections, longevity, high altitude sickness, neural disorders, in addition to increasing physical endurance and work productivity (*Brown* et al., 2002). The demand for products containing *R. rosea* is high. They are mainly taken to reduce physical fatigue and increase mental performance (*Blomkvist* et al., 2009). Studies focusing on roseroot-based products demonstrated its positive impacts on nervous-, heart- and vascular system disorders as well as its anti-fatigue, antidepressant and sedative effects. As a dietary supplement it is used worldwide (*Panossian* et al., 2010).

Rhodiola rosea belongs to the family of Crassulaceae, to the subfamily Sedoideae and to the genus *Rhodiola* (*Ohba*, 1981). *Rhodiola rosea* is a dioecious plant that reaches a height of 30 to 80 cm and produces yellow flowers (*Khanum* et al., 2005) from April to August (*Tasheva & Kosturkova*, 2012). It is a perennial plant with thick rhizome which has a rose-like fragrance when cut (*Khanum* et al., 2005). The average weight of the rhizomes is 70–400 g, but a weight of 3.5 kg can be reached. Leaves are oblong and elliptical in shape (*Tasheva & Kosturkova*, 2012). The genus *Rhodiola* originated from mountainous areas of South-West China and the Himalayas (*Ohba*, 1989). *Rhodiola rosea* is distributed in high mountains and in subpolar regions of the northern hemisphere (*Hegi*, 1963).

Due to the intensive collection of its roots and rhizome the species has become extinct in some native habitats while in others the size of its population has been greatly reduced (*Chiorghita* et al., 2011). The increased demand for its herbal drugs and decline of its natural populations justified the cultivation of *R. rosea* (*Galambosi*, 2005). The first steps during its breeding work include the collection and characterization of the naturally occurring genotypes morphologically and phytochemically. Inter-and intrapopulation genetic diversity can be described by molecular markers.

The DNA based markers are DNA segments that can be used for detecting differences in the genome and are not necessarily manifested in the phenotype (*Agarwal* et al., 2008). Molecular markers also provide an opportunity to assess genetic diversity: recently, they have played an essential role in studying variability, diversity (*Behera* et al., 2008), and in various breeding programs (*Gupta* et al., 2001). Microsatellites or Simple Sequence Repeats (SSRs) are tandem repeat motifs of 1–6 bases, which are present in prokaryotic and eukaryotic genomes similarly, both in coding and non-coding regions (*Wu* et al., 1994; *Agarwal* et al., 2008). Microsatellites have an important role among the DNA based markers due to their abundance and hyper variability (*Gupta* et al., 1996). These markers are well-reproducible and show co-dominant inheritance and multi-allelic properties (*Agarwal* et al., 2008).

In case of *R. rosea* the number of SSR markers is limited. In 2009 *Zini* et al. developed eight microsatellite (SSR) primers specific to *R. rosea*. Four of these have been

used by *Kylin* (2010) studying Scandinavian roseroot populations. During the work of *György* et al. (2014) only four out of these eight SSR markers proved to be informative. *You* et al. (2013) have published SSR markers, but those were not developed for roseroot, but for other *Rhodiola* species. The four most polymorphic was chosen and tested on *R. rosea* by *György* et al. (2014) and two were found to be applicable on that species. However this small number of SSR markers does not provides sufficient genetic resolution for population genetic studies. Therefor the aim of our work was to develop new, *R. rosea* specific SSR markers.

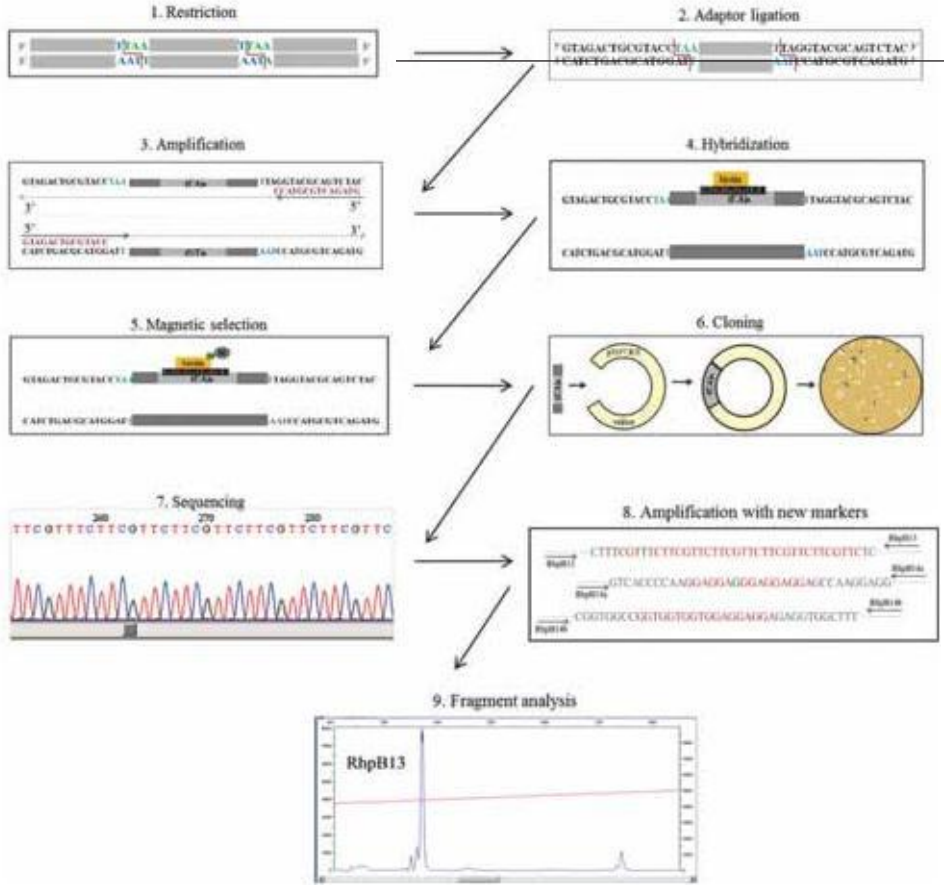

Figure 1. The protocol of the FIASCO method

Materials and methods

Plant material

Plant material originated from the collection of the Department of Genetics and Plant Breeding of Corvinus University of Budapest. The plant material was collected in Austria, Norway, Italy, Russia, Switzerland and Slovenia (*Table 1*) and was identified by collegues from the Department of Botany, CUB.

DNA extraction

DNA was extracted from frozen leaves of *R. rosea* (2/183, 2/186, 2/187, 2/189, M3, M13, M15, S5, S11, S15) using E.Z.N.A. SP Plant Mini Kit (Omega, VWR, Budapest, Hungary) according to the manufacturer's instructions. DNA quality and quantity was measured respectively by ethidium-bromide stained 1% TBE agarose gel electrophoresis and NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies, Bioscene, Budapest, Hungary).

FIASCO (Fast isolation by AFLP of sequences containing repeats)

The development of microsatellite markers was executed by the FIASCO (Fast Isolation by AFLP of Sequences COntaining repeats) method described by *Zane* et al. (2002) and *Bloor* et al. (2001) and shown in *Figure 1*. In the first step (*Figure 1*. 1. step) the S5 (Triglav, Slovenia) genomic DNA sample was cleaved with *Tru1*I restriction endonuclease. The reaction mixture contained 1×buffer R (Fermentas, Biocenter, Szeged, Hungary), 1 U *Tru1*I restriction enzyme (Fermentas, Biocenter, Szeged, Hungary), 8 µl (ca. 2 µg) genomic DNA in a final volume of 20 µl. After the digestion DNA was purified using chloroform, precipitated with 7.5 M ammonium acetate and finally the DNA was dissolved in 15 µl of distilled water.

Adaptors *Tru1*I top and *Tru1*I bottom (see nucleotid sequence in *Table 2*, *Figure 1*. 2. step) were ligated to the restriction enzyme generated sticky ends. Ligation was performed in a final volume of 20 µl containing 10 µl 2×buffer, 2 µl *Tru1*I adaptor, 5 U T4 DNA ligase (Fermentas, Biocenter, Szeged, Hungary), 5 µl digested and purified DNA and 2 µl sterile water. The ligation lasted for 1 hour at 25°C and was followed by chloroform purification as previously described. The resulting fragments were amplified by PCR (Swift MaxPro, Esco Micro Pte. Ltd, Singapore). The 20 µl reaction volume contained 10×PCR buffer, 2.5 mM $MgCl_2$, 0.02 mM dNTP mix, 2.5 mM *Tru1*I top, adaptor-specific primer, 0.5 µl BSA (1 mg/ml), 0.5 U *Taq* DNA polymerase (Fermentas, Biocenter, Szeged, Hungary), 1.5 µl digested DNA and 11.64 µl distilled water (*Figure 1*. 3. step). The following thermal profile was used: initial denaturation at 94°C for 5 min, followed by 25 cycles of 94°C for 30 sec, 52°C for 30 sec and 72°C for 45 sec and a final extension at 72°C for 7 min.

Selection of the $(CA)_n$ containing fragments was carried out according to *Bloor* et al. (2001) (*Figure 1*. 4. step). The reaction components included 2.5 µl of 10 µM 5' biotin-labelled $(GT)_5$ oligonucleotide, 6 µl amplified product

and 11.5 µl sterile water. The mixture was heated to 94°C for 7 min, the DNA was denatured then the temperature was decreased by 0.1°C/sec ramping speed to promote the hybridization of biotin-labelled fragments to the complementary strands. In a subsequent step (*Figure 1*. 5. step), the resulting product was subjected to magnetic selection. At the beginning of the process, pre-washing and pre-blocking steps were carried out. First, 10 µl streptavidin-coated magnetic beads (Dynabeads M-280 streptavidin, Life Technologies Corporation, Invitrogen, Carlsbad, California) was added into a 1.5 ml centrifuge tube and mixed thoroughly. In a second step the tube was placed next to a magnetic stand for 2 min to allow the beads to migrate to the side of the tube, and the supernatant was removed. In the third step 20 µl 1×Washing/Binding (W/B) buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 M NaCl) was added and magnetic beads were suspended. The second and the third step were repeated. Next, 20 µl 2×W/B buffer and 20 µl inert PCR products were measured into the tube. After 5 min the tube was placed to a magnetic stand for 2 min and the supernatant was removed. After these pre-washing and pre-blocking steps 20 µl 2×W/B buffer, 10 µl cleaved DNA with adaptors, 2 µl inert PCR products and 8 µl distilled water were added to the pellet. All was suspended for 15 min then placed to the magnetic stand for 2 min followed by aspiration of the supernatant. Other 37 µl 1×W/B buffer and 3 µl inert PCR products were measured into the centrifuge tube. The solution was swirled for 5 min then the tube was put into the magnetic pole again. After removing the supernatant, 37 µl 1×W/B buffer and 3 µl inert PCR products were added to the system and the tube was shaken for 20 min. The tube was placed into the magnetic column for 3 min and the transparent liquid was removed. Finally, the content of the tube was dissolved in 25 µl TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) buffer and 2.5 µl distilled water. In the next step, the resulted fragments were amplified by the previously described PCR. The following components were mixed in a final volume of 40 µl: 4 µl 10×buffer (Fermentas, Biocenter, Szeged, Hungary), 1.9 µl 25 mM $MgCl_2$, 0.8 µl 10 mM dNTP mix, 6.4 µl 10 µM *Tru1*I top primer, 1 µl BSA (1 mg/ml), 1 U *Taq* DNA polymerase (Fermentas, Biocenter, Szeged, Hungary), 3 µl DNA of the selected fragments and 21.9 µl distilled water. The PCR program consisted of denaturation for 6 min at 94°C, followed by 30 cycles of 94°C for 30 sec, 52°C for 30 sec and 72°C for 45 sec and the final extension at 72°C for 30 min. The success of the reaction was evaluated by gel electrophoresis.

DNA sequencing

The amplified DNA fragments were ligated into pTz57R/T vector using InsTA Clone PCR Cloning Kit (Fermentas, Biocenter, Hungary) (*Figure 1*. 6. step). The ligated plasmids were transformed into DH5 competent cells and blue-white screen technique (*Padmanabhan* et al., 2010) was applied. Plasmid DNA was isolated from recombinant colonies using E.Z.N.A. Plasmid Mini Kit (Omega, CA, USA) and cleaved with *Xba*I and *Bam*HI to determine the presence of the insert. The reaction mixture contained 10×buffer FD (Fermentas, Biocenter, Hungary), 1 U *Xba*I, 1 U *Bam*HI restriction enzymes (Fermentas, Biocenter, Hungary), 2 µl

Table 1. Plant material

Code	**Origin**
2/183	Northern-Norway
2/186	Northern-Norway
2/187	Northern-Norway
2/189	Northern-Norway
9/134	Northern-Norway
9/134	Northern-Norway
10/111	Northern-Norway
A3	Prabichl, Austria
B2	Binntal, Switzerland
B3	Binntal, Switzerland
M13	Mattmark, Switzerland
M15	Mattmark, Switzerland
M3	Mattmark, Switzerland
N1/65	Northern-Norway
N2	Val de Nomnom, Switzerland
P2	Piano dei Canali, Switzerland
S10	Triglav, Slovenia
S11	Triglav, Slovenia
S15	Triglav, Slovenia
S19	Triglav, Slovenia
S20	Triglav, Slovenia
S5	Triglav, Slovenia
U2/1	Sibay, Russia
U4/5	Novaja Zemlja, Russia
U4/5	Novaja Zemlja, Russia
U4/5	Novaja Zemlja, Russia
Ua2	Unteralp, Switzerland
V2	Val Fredda, Italy

Table 2. Sequences of the used and designed primers

Primer	Sequence:
RhpB13 forward:	5'GTAAAACGACAACCAGTCTCAGCCCGCT GCT 3'
RhpB13 reverse:	5'TGTCGGAGAGATGAGTGGTG3'
RhpB14a forward:	5'GTAAAACGACGGCCAGTCAAGCTAGGTC TCA 3'
RhpB14a reverse:	5'TAACCAAGGGAGGAACACCA3
RhpB14b forward:	5'GTAAAACGACGGCCAGTCAAGTGTCCCA TTG3'
RhpB14b reverse:	5'GTGACCGGTTGACCTAGCTT3'
M13*primer:	5'GTAAAACGACGGCCAGT3'
Tru1I bottom:	5'TAGGTACGCAGTCTAC3'
Tru1I top:	5'CTCGTAGACTGCGTACC3'

plasmid (ca. 100 ng/µl) in a final volume of 20 µl. First, the mixture was incubated at 37°C for 15 min, then at 65°C for 10 min. After gel electrophoresis the nucleotide sequences of positive samples were determined by BAYGEN Capillary Sequencing Platform. The resulting chromatograms were visualized using BioEdit (*Hall*, 1999) program (*Figure 1*. 7. step).

PrimerS were designed for microsatellite-containing DNA sequences by Primer3 Software v. 0.4.0 program (*Untergrasser* et al., 2012; *Koressaar & Remm,* 2007). The optimal annealing temperature of the primers were determined by gradient PCR. Two µg genomic DNA (B2) was used for gradient PCR amplification in 20 µl reaction volume containing 2 µl Green*Taq* 10×buffer (Fermentas, Biocenter, Szeged, Hungary), 0.4 µl 10 mM dNTP mix, 0.5 µl 10 µM forward and 0.5 10 µM reverse primer, 0.15 U Dream*Taq* DNA polymerase (Fermentas, Biocenter, Szeged, Hungary) and 15.45 µl distilled water. The PCR thermal profile was the same as previously described exept the different primer annealing temperature.

At last, the genetic variability of the developed microsatellite markers was studied on different individuals of *R. rosea* populations (*Figure 1*. 8. step). The 20 µl PCR mixture contained 2 µl Dream*Taq* 10×buffer (Fermentas, Biocenter, Szeged, Hungary), 0.4 µl 10 mM dNTP, 0.4 µl 10 µM forward, 0.5 µl 10 µM reverse and 0.3 µl 10 µM M13* primer (*Table 2*), 0.15 U Dream*Taq* DNA polymerase (Fermentas, Biocenter, Szeged, Hungary), ca. 2 µg genomic DNA and 15.25 µl distilled water. The M13* PCR primer is 5' FAM fluorescent labelled oligonucleotide, which is needed to detect the size of the DNA fragments. The thermal profile of the PCR was the same as above. On the basis of gel electrophoresis the selected fragments were run in an automated sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The obtained chromatograms were evaluated by Peak Scanner Software v 1.0 program (Applied Biosystems, Foster City, CA, USA) (*Figure 1*. 9. step).

Results

The aim of this study was to develop novel microsatellite markers for *R. rosea.* The concentration of the isolated DNA was between 100–200 ng/µl. According to the agarose gel electrophoresis the best quality DNA was the S5 (135,7 ng/µl). This was chosen and digested with *Tru1*I restriction enzyme. Adaptors were ligated to the sticky ends allowing the resulting fragments to be amplified (*Figure 2*). The $(CA)_n$ containing fragments were selected by magnetic selection based on biotin-streptavidin linkage. Following the cloning of the resulting products one hundred six colonies were checked by colony PCR. The positive samples were selected based on gel electrophoresis. In those cases where amplification occurred, different sizes of DNA fragments were detected. Forty-four of the positive colonies were propagated in liquid culture and plasmids were isolated from them (*Figure 3)*. To ensure that inserts of different size were cloned the isolated plasmids were cleaved. Finally, sequence analysis of forty-three plasmids was carried out.

Out of the forty-three sequenced samples only three contained microsatellite regions, however neither of them contained the aimed $(CA)_n$ repeats. The identified loci were named as RhpB13 $(TTGATTC)_5$, RhpB14a $(GGA)_5$ and RhpB14b $(GGT)_3(GGA)_3$. Primer pairs were developed for the flanking regions of these 3 microsatellites (*Table 2*). Applying 53°C annealing temperature all three loci could be amplified (*Figure 4*). For RhpB14b primer pair 53°C proved to be satisfactory, but in the case of RhpB14a and RhpB13 markers the amplification was weak so further optimization was carried out by gradient PCR with V2 genomic DNA. Finally, 63°C as the optimal annealing temperature (*Figure 5*) was chosen. For RhpB13 optimal reaction conditions could not be determined.

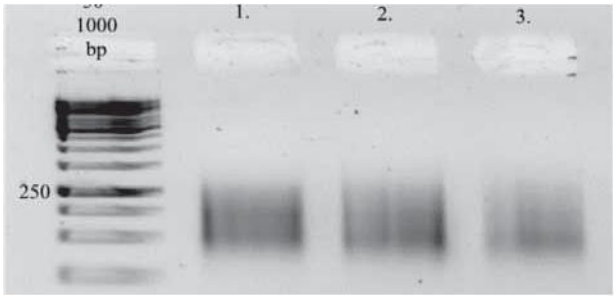

Figure 2. Electrophoretic gel photo of the PCR products after the digestion with Tru1I restriction endonuclease followed by adaptor ligation. First lane is 50 bp Gene Ruler (Fermentas, Biocenter, Hungary). 1-3 lanes mean different amount of template DNA: 0.75 µl, 1.5 µl and 2.5 µl. As a result, many, different fragments were amplified. The work was continued with the second sample.

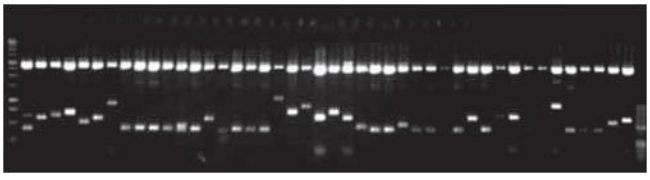

Figure 3. Restriction cleavage of plasmids purified from transformant colonies. First lane is 1 kB Gene Ruler (Fermentas, Biocenter, Hungary) and the last lane is 50 bp Gene Ruler (Fermentas, Biocenter, Hungary). Plasmids are about 3000 bp while the inserts have different lengths. Out of forty-four plasmids forty-three were send to sequence analysis.

In the last step of the marker development the designed primers were tested on several *R. rosea* specimens originated from different sites. The sizes of the amplified microsatellite fragments were determined by fragment length analysis. The amplification was sufficient to give information on the polymorphism of the locus, but not with the desired reliability. *Rhodiola rosea* is a diploid, outcrossing species and as such one or two different microsatellite alleles were expected with high proportion of heterozygote individuals. *Table 3* shows that in case of some samples three fragments were detected. This means that the primer pairs annealed to multiple loci. Based on the fragment length analysis RhpB14a amplified

the same fragment sizes in each sample meaning that the locus is not polymorphic. With primers designed for both RhpB14b and RhpB13 three fragments were amplified in most of the cases. One of these was constantly present, while size variability was observed in the other two fragments. The performance of the RhpB14b and RhpB13 markers should be further improved, but two novel polymorphic markers were found in the genome of *R. rosea*.

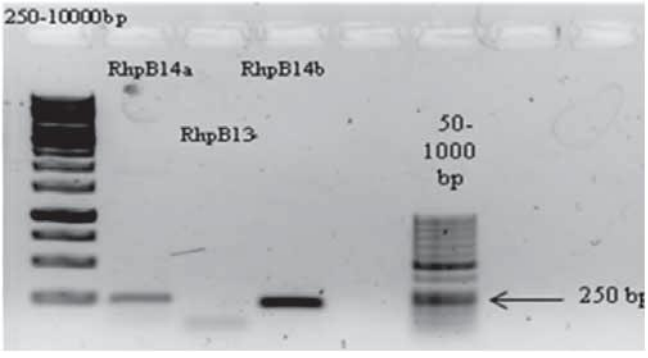

Figure 4. Test PCR with the designed primers at 53°C. The amplification with RhpB14b was satisfactory, but the other two needed to be optimised

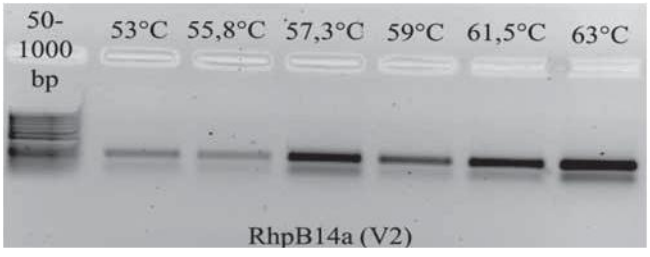

Figure 5. Gradient PCR of RhpB14a primer with V2 genomic DNA sample. First lane is 50 bp Gene Ruler (Fermentas, Biocenter, Hungary). The amplified fragments were at 250 bp with different annealing temperature of the primer

Table 3. Result of the fragment length analysis

DNA	**RhpB13 primer (bp)**		
N1/65	237	**223**	272
U4/5	216	**223**	258
S10		**223**	258
S5	237	**223**	272
U4/5	151	**223**	160
U2/1	216	**223**	
DNA	**RhpB14a primer (bp)**		
9/134	247	**238**	
B2	247	**238**	
S19	247	**238**	232
Ua2	247	**238**	229
P2	247	**238**	229
N2	247	**238**	
DNA	**RhpB14b primer (bp)**		
9/134	180	**214**	244
A3	209	**214**	
B3	180	**214**	189
S20	203	**214**	248
U4/5	180	**214**	197
10/111	203	**214**	

Discussion

Today microsatellites are the most widely used molecular markers. They have several advantages compared to dominant markers like RAPD or AFLP, namely the codominant nature, locus specificity, good reproducibility and high polymorphism (*Powell* et al., 1996). The main limitation of the application of microsatellite technology is indeed its locus specificity. Microsatellite regions need to be isolated *de novo* from species being examined for the first time (*Sudheer* et al., 2010). The method of fast isolation by AFLP of sequences containing repeats (FIASCO) published by *Zane* et al. (2002) is the most widespread technique.

Zini et al. (2009) developed 8 microsatellite markers for *R. rosea* by the FIASCO method. In their work out of ninety-six colonies, thirty-seven contained microsatellite loci. Out of twenty three primer pairs designed 15 amplified DNA fragments but three Markers were monomorphic and four Markers pronounced multi-banding pattern. The further eight microsatellite loci were polymorphic. As comparison, to the work of *Zini* et al. (2009) out of the forty-three sequenced clones three contained microsatellite regions in our study. One of them was monomorphic but the other two were polymorphic.

Since the results at each check point were in line with the expectations, theoretically all the samples should have contained tandem repeat motives. The unexpectedly low proportion of microsatellite-containing sequences might be due to the fact that the applied oligonucleotide labelled with biotin was too short, hence hybridized also to stretches that did not contain $(CA)_n$ regions. The possible solution to overcome this problem may be a longer oligonucleotide labelled with biotin: *Sudheer* et al. (2010) used oligonucleotides containing the dinucleotide motif 15-times or *Zhang* et al. (2008) used oligonucleotids containing dinucleotides repeated 17 times or trinucleotides repeated 12 times. However, the drawback of such solution would be that the genome contains considerably lower number of complementary sequences for longer oligonucleotides; therefore the hybridization would occur at a lower rate. Another possibility is to use two biotinylated oligonucleotids simultaneously during the enrichment process similarly to *Sudheer* et al. (2010).

A further possible cause of the low proportion of microsatellite-containing sequences may arise from not fully appropriate conditions for magnetic-selection, these are, however, difficult to be optimized. *Yang* et al. (2009) used the desired microsatellite motif as primer in the colony PCR, thus ensuring a more sensitive selection. Another way to increase the number of microsatellite-containing fragments could be used Southern blot following the colony PCR, which should be performed using radioactive labelled probes, thus ensuring a more sensitive selection.

Since the number of microsatellites occurring in the genome depends on the size of the genome of the organism (*Tóth* et al., 2000), which is not known in case of *R. rosea*, it is difficult to draw reliable conclusions with regard to the success rate of our study.

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