Acta fytotechn. zootechn., 18, 2015(4): 83–89

Original Paper

Application of the RAPD and miRNA markers in the genotyping of *Silybum marianum* (L.) Gaertn

Katarína Ražná*, Lucia Hlavačková, Milan Bežo, Jana Žiarovská, Miroslav Habán, Zuzana Sluková, Mária Pernišová

Slovak University of Agriculture in Nitra, Slovak Republic

Two types of molecular markers based on randomly amplified DNA by RAPD-based assay and amplified microRNA by miRNA-based assay were applied for the genotyping of five accessions of *Silybum marianum* (L.) Gaertn. It was also verified the effectiveness of the isolation of genomic DNA by three commercially available isolation kits and two similar complex methods and their applicability for this medicinal herb. None of the commercial isolation kits provided the genomic DNA of sufficient quality and quantity. Applied originally designed CTAB method and modified CTAB method for purpose of DNA isolation from medicinal plants allowed to isolate DNA of required quality and of sufficient yield. RAPD-based DNA fingerprints, allowed to distinguish the individual genotypes of *Silybum marianum*. MicroRNA-based markers showed the cross-genera transferability potential and displayed sufficient level of polymorphism. Both types of molecular markers could be used as suitable tool for genotyping of milk thistle. However, because of the size of miRNA amplicons is the efficiency of miRNA-based markers PCR amplifications preferred and consequently less DNA quality-dependent which may be advantageous in view of the content of undesirable secondary metabolites in medicinal plants.

Keywords: molecular markers, RAPD, miRNA, *Silybum marianum*

1. Introduction

Silybum marianum L. Gaertn., or milk thistle, is a medicinal plant of unique pharmaceutical properties for treating liver diseases, used as a cytoprotectant, anticarcinogen and for the treatment of diabetic nephropathy (Rafieian-Kopaie and Nasri, 2012; Mohammadi et al., 2011; Rainode, 2005). The active complex of milk thistle is a lipophilic extract from plant seeds and it is composed of three isomer flavonolignans (silybinin, silydianin and silychristin) collectively known as silymarin. Silybin is a component with the greatest degree of biological activity and it makes up 50% to 70% of silymarin. Silymarin is found in the entire plant but it is concentrated in the fruit and seeds. Silymarin acts as an antioxidant by reducing the production of free radicals and peroxidation of lipids, has antifibrotic activity and may act as a toxin blockade agent by inhibiting the binding of toxins to the hepatocyte cell membrane receptors (Abenavoli et al., 2010). *Silybum marianum* is the most cultivated medicinal plant in Slovakia. In the years 2014–2015 it exceeded the growing area of 1000 hectares. The long-term interest in the cultivation of this medicinal plant is significant in terms of its silymarin complex for the production of pharmaceuticals (Habán and Vaverková, 2014; Habán et al., 2015).

The determination of genetic diversity within and among the populations is of a great importance for the improvement of medicinal plants (Mohammadi et al., 2011). Futhermore, the identification of genetic relationships among the populations or genotypes is essential for the efficient utilization of the genetic resources of this medicinal plant. On the other hand, the unequivocal identification and authentication of the herbal plants used for production is a critical step at the beginning of an extensive process of quality assurance. The DNA-based molecular markers play a significant role in gene mapping, genetic diversity analysis, germplasm evaluation and molecular marker-assisted selection (Fu et al., 2013). Genomic fingerprinting can differentiate between individuals, species and populations and it is useful for the detection of homogeneity of the samples and presence of adulterants. The generation of molecular "barcodes" of medicinal plants will be worth the concerted effort and contribute to the ongoing effort of defining the barcodes for every species on earth (Sucher and Carles, 2008).

RAPD can amplify the random fragments of DNA using the primer pairs with arbitrary nucleotide sequences (Williams et al., 1990). The PCR yields a mixture of amplified fragments (amplicons) of various sizes that can

***Correspondence:** Katarína Ražná, Slovak University of Agriculture in Nitra, Faculty of Agrobiology and Food Resources, Department of Genetics and Plant Breeding, Tr. Andreja Hlinku 2, 949 76 Nitra, Slovak Republic, e-mail: katarina.razna@uniag.sk

Faculty of Agrobiology and Food Resources, 2015
http://www.fapz.uniag.sk/
DOI: 10.15414/afz.2015.18.04.83–89

be analyzed by electrophoresis (Sucher and Carles, 2008). The patterns obtained with the specific DNA sample are its "fingerprint".

The RAPD marker analysis used in the study by Sharaf et al. (2010) with the aim to characterize 12 *Silybum marianum* accessions resulted in 83 DNA fragments, 33 of which were polymorphic and ten accessions proved to have specific molecular markers. Ten random primers out of 15 gave reproducible results. The RAPD markers are dominant, as DNA segments of the same length are amplified from one individual but not from another (Williams et al., 1990). It is not possible to recognize whether a DNA segment is amplified from a locus that is heterozygous (1 copy) or homozygous (2 copies). Another type of the PCR-based technique, amplified fragment length polymorphism (AFLP), was employed to investigate the population of 32 Iranian *Silybum marianum* populations along with two commercial varieties (Mohammadi et al., 2011). A total of 415 polymorphic marker loci were produced by 27 primer combinations. Similar to the RAPD markers, even the AFLP markers are a dominant type of markers.

The inherent problems of reproducibility make the RAPDs unsuitable for the transference or comparison of results among the research teams working with similar species and subjects (Kumar et al., 2009). Although RAPD is suitable for the rapid sample authentication as well as the assessment of sample purity, it is often not easy to replicate the fingerprint patterns established in one laboratory because even slight (instrumentation-dependent) variations during the PCR can result in variant fingerprints even when the samples of the same genomic DNA are used (Sucher and Carles, 2008).

Fu et al. (2013) developed a new type of molecular markers based on microRNA with high reproducibility, sufficient polymorphism and high efficiency of production. MicroRNAs are a type of endogenous non-coding RNAs prevalent in the genomes of many organisms. The high conservation of miRNA and pre-miRNA sequences provides an opportunity to develop a novel molecular marker type. MicroRNAs (miRNAs) have recently emerged as important regulators of gene expression in plants (Galla et al., 2013). MiRNAs are 21–24 nt RNA sequences derived from the single-stranded RNA precursors, which possess the ability to form intra-molecular complementary hairpin structures. This feature distinguishes miRNAs from other sRNAs, such as small interfering RNAs, which originate from double-stranded RNAs derived from the inter-molecular hybridization of two complementary RNA molecules (Bej and Basak, 2014; Barvkar et al., 2013; Gursanscky et al., 2011). The application of miRNA as a novel type of genetic markers has been developed for genotyping applications in foxtail millet (*Setaria italica* L.) and related grass species (Yadav et al., 2014), in combination with the SSR markers in *Oryza sativa* (Mondal and Ganie, 2014) with the aim to identify the salt responsive miRNA-SSR markers and in the genotyping of *Brassica* species (Fu et al., 2013). The application of those markers based on miRNA has not been used in genotyping within a single plant species.

Considering the above, this study attempted to establish the miRNA-based molecular markers system for *Silybum marianum* L. Gaertn. genotypying applications and its comparison with the DNA-based molecular markers, RAPD.

2. Material and methods

2.1 Materials and DNA extraction

A total of 5 genotypes of *Silybum marianum* L. of various origins were used in this study: Silyb 1 (Malanta, Slovak Republic), Silyb 2 (Šumperk, Czech Republic), Mirel (Brno, Czech Republic), Silma (Poland) and a sample of unknown origin (SM). The samples in the present study are labeled from 1 to 5. The biological material was cultivated on the MS (Murashige-Skoog) culture medium in *in vitro* conditions in the 16/8 (light/dark) photoperiod. Based on the original methodology of this type of experiment, we have used as a starting material for all types of primers, the leaves of seedlings. The total genomic DNA was extracted from the leaves of 10-day old seedlings using three different commercial plant DNA isolation kits and two complex methods, the cetyltrimethylammonium bromide (CTAB) method described by Rogers and Bendich (1994) and the modified CTAB extraction applied for medicinal plants by Padmalatha and Prasad (2006). The extracted DNA was quantified by NanoPhotometer™ (IMPLEN) and diluted then into 50 ng μl^{-1} with nuclease-free water (Fermentas) for PCR amplification.

2.2 Marker assay

PCR-RAPD amplification reactions in a 25 µl reaction mixture contained 1 unit of i-*Taq*™ DNA polymerase, 0.2 mmol dm^{-3} dNTPs, 1 µmol dm^{-3} of primer (Table 1), 1 mmol dm^{-3} of $MgCl_2$ and 1 × i-*Taq* PCR buffer (20 mmol dm^{-3} Tris-HCl; pH 8.4; 50 mmol dm^{-3} KCl). The DNA amplification protocol was 5 min at 94 °C, followed by 45 cycles of 30 s at 94 °C, 30 s at 36 °C, 1 min at 72 °C and the final 7 min cycle at 72 °C.

The miRNA-based markers were PCR amplified in a 20 µl reaction mixture that contained 2 units of Dream*Taq* DNA polymerase, 0.8 mmol dm^{-3} dNTPs, 10 pmol dm^{-3} of each primer and 1 × Dream*Taq* PCR buffer (KCl; $(NH_4)_2SO_4$; 20 mmol dm^{-3} $MgCl_2$). The combinations of primer pairs used for the miRNA-based marker assay are shown in Table 2. The PCR amplification used the 'touchdown' method is as follows: initial denaturation at 94 °C for 5 min; 5 cycles of 30 s at 94 °C, 45 s at 64 °C (the

DOI: 10.15414/afz.2015.18.04.83–89

Table 1 Sequences of random primers for RAPD marker assay

Primer	Sequences 5´–3´
OPB 03	CATCCCCCTG
OPB 04	GGACTGGAGT
OPB 05	TGCGCCCTTC
OPB 07	GGTGACGCAG
OPB 12	CCTTGACGCA
OPB 13	TTCCCCCGCT
OPB 15	GGAGGGTGTT
OPB 16	TTTGCCCGGA
OPB 17	AGGGAACGAG
OPB 18	CCACAGCAGT
OPB 19	ACCCCCGAAG
OPB 20	GGACCCTTAC

temperature was decreased by 1 °C per cycle), and 60 s at 72 °C; 30 cycles of 30 s at 94 °C, 45 s at 60 °C, and 60 s at 72 °C; and the final extension at 72 °C for 10 min.

The PCR products were separated on 3% agarose gel (RAPD assay), respectively on 15% TBE-Urea gels (miRNA assay) in 1 × TBE Running Buffer at a constant power of 90 V, 120 mA for 60 minutes, respectively at 180 V, 15 mA for 90 min. The polyacrylamide gels were stained with GelRed™ (0.5 μg ml^{-1}, Biotium) and were visualized under UV by the G-Box electrophoresis documentation system (Syngene). The DNA fragments size was compared to 100 bp Gene Ruler (ThermoScientific) and in the case of polyacrylamide gels by 10 bp DNA ladder (Invitrogen).

3. Results and discussion

The molecular markers used in this research were applied in order to obtain the fingerprinting of individual genotypes of *Silybum marianum* L. The DNA-based molecular markers have been used as randomly amplified DNA fragments by the RAPD primers and intergenic DNA amplified fragments by microsatellite sequences-based primers. The RNA-type molecular markers were developed based on the sequences of microRNA.

Table 2 Combination of primer pairs used for miRNA marker assay

Primer	Sequences 5´–3´
gm-miR 156b **gm-miR 171a**	TGACAGAAGAGAGAGAGCACA TGAGCCGTGCCAATATCACGA
gm-miR 156b **lus-miR 168a**	TGACAGAAGAGAGAGAGCACA CACGCATCGCTTGGTGCAGGT
m-miR 171a **lus-miR 168a**	TGAGCCGTGCCAATATCACGA CACGCATCGCTTGGTGCAGGT
hyp-miR 414 **lus-miR 168a**	CACAGCGAAACCCACGAG CACGCATCGCTTGGTGCAGGT

Legend: lus – *Linum usitatissimum*, gm – *Glycine max*, hyp – hypericum perforatum, miR – microRNA

One of the key factors affecting the successful application of the genome markers is the quality of isolated DNA. We have tested three different commercial plant DNA isolation kits and two complex methods, the cetyltrimethylammonium bromide (CTAB) method described by Rogers and Bendich (1994) and the modified CTAB extraction applied for medicinal plants by Padmalatha and Prasad (2006). Commercially available genomic DNA isolation kits are designed for rapid and efficient purification of high quality genomic DNA from wide variety of plant species and tissue types. They apply silica-based membrane technology in the form of a convenient spin column, eliminating the need of toxic phenol-chloroform extractions, or time-consuming alcohol precipitation. Unfortunately, none of the commercial isolation kits provided the genomic DNA of sufficient quality and quantity. Several medicinal plants contain an exceptionally high amount of polysaccharides, polyphenols, tannins, hydrocolloids (sugars and carragenans) and other secondary metabolites such as alkaloids, flavonoids, phenols, terpenes and quinines, which would interfere with the DNA isolation procedures and subsequent downstream applications (Padmalatha and Prasad, 2006). Applied CTAB method by Rogers and Bendich (1994) and modified CTAB method for purpose

Table 3 Comparison of the yield and quality DNA isolated by CTAB-based methods

Sample	DNA yield in ng μl^{-1}	DNA quality	
		A260/280	A260/230
Silyb 1 (1)	393/167*	2.082/1.467*	1.485/1.480*
Silyb 2 (2)	108/109*	1.938/1.396*	0.837/1.91*
Mirel (3)	54/73*	1.969/1.959*	0.933/0.786*
Silma (4)	181/429*	2.041/1.479*	1.273/1.906*
Unknown	122/156*	1.997/1.393*	1.4/1.816*

Legend: numbers 1–5 indicate the samples used in the assay, A – absorbance at certain wavelength, * – indicates the samples DNA isolated by method Padmalatha and Prasad

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DOI: 10.15414/afz.2015.18.04.83–89

of DNA isolation from medicinal plants by Padmalatha and Prasad (2006) allowed to isolate DNA of required quality and of sufficient yield (Table 3). Regarding the efficiency of both CTAB-based methods there was no significant difference in the DNA yield. Concerning the values referring to protein contamination (measured at A260/280) better parameters were recorded in DNA samples isolated by unmodified CTAB method. However, the DNA contamination values referring to the secondary metabolites, measured at the absorbance A260/230, has shown better values by the application of the modified CTAB method by Padmalatha and Prasad (2006).

For the purpose of comparing the quality of isolated DNA on the efficiency of the downstream PCR-based applications, the individual DNA samples were used (Figure 1 and 2). Whereas part of isolated DNA was consumed in the optimization of amplification protocols of both marker assays and because of insufficient quantity of DNA samples no. 3 (Mirel), the sample was not included in all amplification reactions for RAPD marker assay.

3.1 RAPD marker assay

In order to test the quality of isolated DNA as well as the effectiveness of both isolation methods we used for RAPD marker assay samples of DNA originated from both methods. Twelve decamer primers were used in the RAPD reactions, 7 of which were amplified DNA fragments (Figure 1 and 2). 10, 9 respectively 8 DNA fragments were amplified in the primers OPB 07, OPB 04 and OPB 12 in the samples Silyb 1 (Malanta), Silma (Poland) and in the sample of unknown origin (SM). The primers OPB 17 and OPB 18 amplified 7, respectively 6 DNA fragments in total in the samples Silyb 1 and the sample of unknown origin. Four, respectively three DNA fragments have been recorded in the decamers OPB 05 and OPB 03 in the samples Silyb 1, Silma and in the sample of unknown origin.

One possible reason of the unrecorded RAPD amplification profile of the samples Silyb 2 (Šumperk) and Mirel (Brno) is the contamination of the secondary metabolites, which was higher compared to the other samples. Despite the fact that none of the tested primers were capable of amplifying the DNA profile of all tested samples, we can state that in each of them specific DNA fingerprints were recorded. In the primer OPB 07, it was possible to distinguish 3 out of 5 tested samples of milk whistle by the amplification of 10 DNA fragments in total. The size of amplified fragments ranged from 200 bp to 900 bp based on used primer.

3.2 MicroRNA marker assay

The primers applied in this study have been designed and used in the miRNA-based genotyping of other crop species (Hlavačková et al., 2015a; Hlavačková et al., 2015b, in press). For the purpose of this research, the suitability of the primer sequences have been verified by the bioinformatic approach described below.

3.3 Characterisation of selected miRNA

The following types of miRNA were selected for the assay: gm-miR156b, gm-miR171a, lus-miR168, and hyp-miR414. Both types of miRNA in soybean genome, gm-miR156b and gm-miR171a, were selected based on the study by Kulcheski et al. (2010). They compared the expression level of 10 soybean miRNA in order to investigate their expression stability in different soybean tissues and genotypes as well as after the abiotic or biotic stress treatment. They provide evidence that the expression stability of gm-mir156b was the highest across the soybean experiment. The NormFinder analysis, which considers the intra- and intergroup variation of gene expression, identified the gm-miR171a as the top ranking house-keeping gene. The miR168 belongs to the group of potential biomarkers of plant stress response (Bej and Basak, 2014; Neutelings et al., 2012). Under different types of abiotic stress (drought, salinity, cold, UV, mechanical, and heat stress), up-regulation of miR168 expression (Bej and Basak, 2014; Sunkar, 2010)

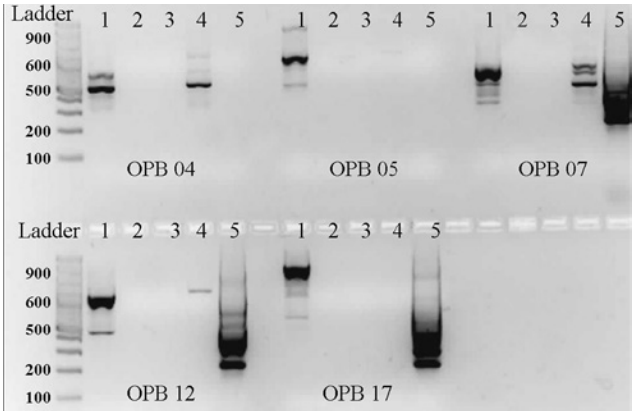

Figure 1 RAPD marker assay of DNA samples isolated by non-modified CTAB method

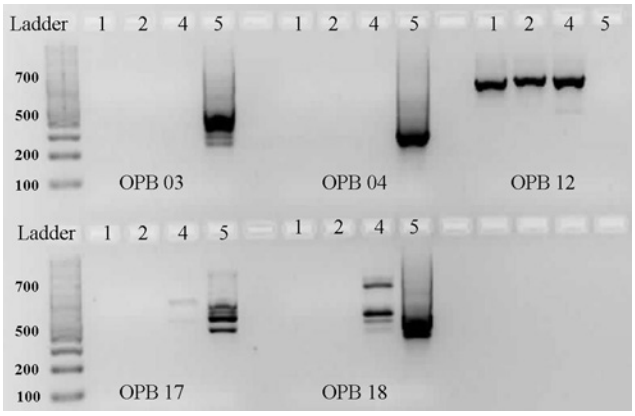

Figure 2 RAPD marker assay of DNA samples isolated by modified CTAB method

DOI: 10.15414/afz.2015.18.04.83–89

has been observed. The expression alterations of lus-miR168 under the excessive use of fertilizers have been studied extensively (Melnikova et al., 2014). Because the record of miRNA sequencing of *Silybum marianum* is not known and the study of these molecules in the genomes of medicinal plants is not very common as in crop plants, we decided to use further primer findings from the study of *Hypericum perforatum*. Hyp-miR414 belongs to the *Hypericum* miRNA families. The *H. perforatum* flowers share highly conserved miRNAs and these miRNAs potentially target dozens of genes with a wide range of molecular functions, including metabolism, response to stress, flower developments and plant reproduction (Galla et al., 2013). The miR414 family was identified as containing the largest number of targets and has proved to be strongly expressed in all flower verticils (i.e., anthers, pistils, sepals and petals), including the young buds, while transcripts of these precursors were neither detected in the leaves nor in the roots.

3.4 miRNA primer design

The primers for the miRNA-based markers were designed according to the mature miRNAs sequences, which are part of the miRNA precursors (pre-miRNA) originated from the miRNA database (http://www.mirbase.org/). Based on botanic classification belongs *Silybum marianum* L. Gaertn. to the family *Asteraceae* and tribe *Cynareae* (Červenka et al., 1986). For the family *Asteraceae*, the miRBase (http://www.mirbase.org/) contains only the sequences of two species, *Cynara cardunculus* and *Heliathus* sp. Since *Silybum marianum* and *Cynara cardunculus* belong to the same botanical tribe, we used the miRNA sequences of *Cynara cardunculus* for the BLAST comparison of selected miRNAs. In the first step, the sequences of *Cynara cardunculus* (cca-miR156b, cca-miR-168a, cca-miR171) were compared to the sequences of selected types of miRNA (mentioned above) by the BLASTn algorithm of the NCBI database (http://blast.ncbi.nlm.nih.gov/). In the second step, we compared those two types of miRNA sequences to the *A. thaliana* mature miRNA (Table 4 and 5). Based on the E-values and the percentage of identities, these primers were considered as suitable for our purposes.

Table 5 Evaluation of BLASTn alignment of miRNA sequences of miRNA-based primers (gm-miR156b, gm-miR171a and lus-miR168a) and selected miRNA of A. thaliana (ath-miR156b, ath-miR168a and ath-miR171a).

miRNA-based primers	miRNA of *A. thaliana*	
	E-value	identities
gm-miR156b	0.004	100%
lus-miR-168a	0.014	100%
gm-miR171a	6.10–11	100%

E – expectancy value

3.5 Marker assay of miRNA-based molecular

MicroRNA marker assay was conducted on DNA isolated according the protocol by Padmalatha and Prasad (2006). As shown by the efficiency of amplification of the marker assays, the DNA quality-dependent RAPD marker assay, has not proven in the case of miRNA markers assay.

A total of 4 miRNA-based molecular marker primers were used. The single primers were randomly recombined together to perform a marker assay (Table 3). Four primer pairs combinations amplified a total of 44 loci. The number of bands produced per primer combination ranged from 4 to 6. The amplified length of PCR products ranged from 40 bp to up to 100 bp. Two primer combinations gm-miR171a/lus-168a and hyp-miR414/lus-miR168a amplified the polymorphic fragments (Figure 3). Although the combination of the additional primers hyp-miR141 and lus-miR168a allowed the amplification of polymorphic fragments, it is likely that the presence of hyp-miR141 is not sufficient in the *Silybum marianum* leaves as it was declared by Galla et al. (2013) in the case of *H. perforatum*. The miR414 family has proved to be strongly expressed in the flowers while the transcripts of these precursors were detected neither in the leaves nor in the roots. The presence of miR414 is probably explicitly tied to the flower organs.

Table 4 Evaluation of BLASTn alignment of miRNA sequences of *Cynara cardunculus* to the sequences of selected miRNA of *A. thaliana* (ath-miR156b, ath-miR168a and ath-miR171a) and miRNA-based primers (gm-miR156b, gm-miR171a and lus-miR168a)

Cynara cardunculus miRNA	miRNA of *A. thaliana*		miRNA-based primers	
	E-value	identities	E-value	identities
cca-miR156b	9.10^{-10}	100%	2.10^{-7}	95%
cca-miR168a	6.10^{-11}	100%	6.10^{-11}	100%
cca-miR171	1.10^{-8}	95%	9.10^{-4}	88%

E – expectancy value

Faculty of Agrobiology and Food Resources, 2015
http://www.fapz.uniag.sk/
DOI: 10.15414/afz.2015.18.04.83–89

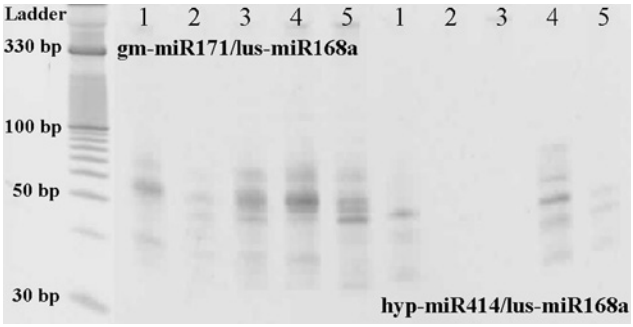

Figure 3 miRNA marker assay of *Silybum marianum* samples by primers combinations gm-miR171a/lus-miR168a and hyp-miR414/lus-miR168a

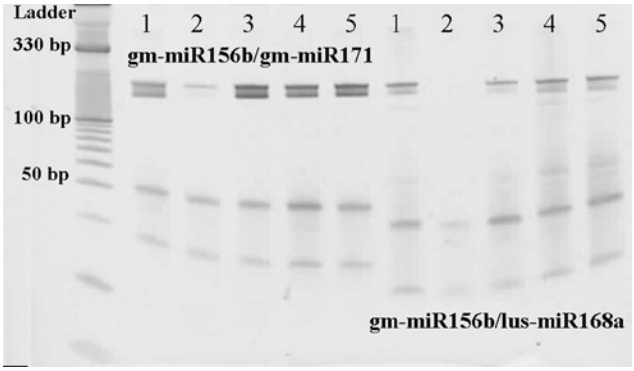

Figure 4 miRNA marker assay of *Silybum marianum* samples by primers combinations gm-miR156b/gm-miR171 and gm-miR156b/lus-miR168a

Monomorphic fingerprint profile of five analyzed milk thistle genotypes was observed by application of primer pairs combinations gm-miR156b/gm-miR171 and gm-miR156b/lus-miR168a (Figure 4). The number of bands produced per primer combination ranged from 4 (gm-miR156b/gm-miR171) to 7 (gm-miR156b/lus-miR168a). The amplified length of PCR products ranged from 30 bp to almost 200 bp. Referred to two pairs of primers, these allow the amplification of specific DNA profile but, for the purpose of genotyping are not useful combinations. For the genotyping of 16 accessions of the six *Brassica* species (Fu et al., 2013) was applied 15 miRNA-based primer pairs which amplified a total of 56 loci. The number of bands produced per primer combination ranged from two to eight and the amplified length of most PCR products ranged from 150 bp to 500 bp.

Based on the obtained DNA fingerprints is possible to point out the cross-genera transferability potential on miRNA-based molecular markers. Basically three types of species-specific miRNA, originated from the genome of *Linum usiatatissimum*, *Glycine max* and *Hypericum perforatum* were used for primers design. Similar results were ontained by Yadav et al. (2014) where the primers for genotyping applications were developed based on miRNA of the model crop foxtail millet. Those primers reflected good transferability across other related grasses including rice, maize, wheat, sorghum and *Brachypodium*.

4. Conclusions

For the purposes of molecular analysis of medicinal plants was the quality and quantity of isolated DNA comparable in both extraction methods used in this study. Based on the type of random primers applicable for RAPD markers-obtained DNA fingerprints, it is possible to distinguish the individual genotypes of *Silybum marianum*. MicroRNA-based primers showed the cross-genera transferability potential. These findings indicate that the miRNA-based molecular markers displayed sufficient level of polymorphism in analyzed *Silybum marianum* genotypes and could be used as suitable genotyping technique to differentiate within genotypes of one specimen.

5. Acknowledgement

This work has been supported by the Excellence Center for Agrobiodiversity Conservation and Benefit project implemented under the Operational Programme Research and Development financed by European Fund for Regional Development (ITMS:26220120015), by the project of Slovak Research and Development Agency APVV-0740-11 Understanding of Plant Adaptation in the Radioactive Chernobyl Area, and co-funded by European Community under project no 26220220180: Building Research Centre "AgroBioTech".

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Faculty of Agrobiology and Food Resources, 2015
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