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ANALYSIS OF STABILITY OF TRINUCLEOTIDE TTC MOTIFS IN COMMON FLAX PLANTED IN THE CHERNOBYL AREA

Veronika Lancíková[1], Lucia Hlavačková[2], Jana Žiarovská[2], Hana Kubíková[2], Milan Bežo[2], Katarína Ražná[2], Maksym Danchenko[3], Namik Rashydov[3], Martin Hajduch[1]*

Address(es):
[1]Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Akademická 2, 950 07 Nitra, Slovak Republic
[2]Slovak University of Agriculture in Nitra, Faculty of Agrobiology and Food Resources, Department of Genetics and Plant Breeding, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic
[3]Institute of Cell Biology and Genetic Engineering in Ukraine, 148 Academika Zabolotnoho St. Kiev, Ukraine

*Corresponding author: jana.ziarovska@uniag.sk

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ABSTRACT

Flax *(Linum usitatissimum* L.*)* is one of the oldest domesticated plants — it was cultivated as early as in ancient Egypt and Samaria 10,000 years ago to serve as a source of fiber and oil, whence it later spread around the world. Compared with other plants, the flax genome consists of a high number of repetitive sequences, middle repetitive sequences and small repetitive sequences of nucleotides. The aim of the study was to analyze the stability of the existing trinucleotides motifs of microsatellite DNA of the flax genome (genotype Kyivskyi), growing in the Chernobyl conditions. The Chernobyl area is the most extensive "natural" laboratory suitable for the study of radiation effects. Over the last 20 years, the researches collected important knowledge about the effects of low and high radiation doses on the DNA isolated from the plant material growing on the remediated fields near Chernobyl and the plant material from fields contaminated by radioactive cesium ^{137}Cs and strontium ^{90}Sr. Using eight pairs of microsatellite primers, we successfully amplified the samples from the remediated fields. For each primer in the control samples and remediated samples, we detected 1 to 3 fragments per locus, each in size up to 120 to 250 base pairs. The applied microsatellite primers confirmed the monomorphic condition of microsatellite loci.

Keywords: Flax, *Linum usitatissimum* L., microsatellites, PCR

INTRODUCTION

Flax (*Linum usitatissimum L.)* is an annual herb, the third largest natural fiber crop and one of the five major oil crops in the world **(Millam *et al*., 2005)**. Flax, which is primarily grown as an oilseed crop for food, feed and in bio-product applications, is a small-sized and self-pollinated herb. Most oilseed flax varieties are rich in the omega-3 (alpha linolenic acid, 55–57 %) fatty acid, which has been functionally associated with numerous health claims **(Cloutier, 2009)**. Linseed oil is used in the production of paint, soap, putty and polymers. The fibers are used in textile, automobile and construction industry, while flax seeds are used mainly in food industry **(McDill *et al*., 2009).** The improvement, maintenance, evaluation and utilization of flax germplasm are nowadays the most important direction in the flax industry **(Zhuchenko *et al*., 1996; Bačelis 2001)**. Flax has a small estimated genome size of ~370 Mbp **(Cloutier, 2012)** and is well suited for fast progress in genomics. Different molecular marker techniques have been applied in the flax molecular marker development and genetic resource evaluation (RAPD, RFLP, AFLP, SSR) **(Spielmeyer *et al*., 1998; Oh *et al*., 2000; Roose-Amsaleg *et al*., 2006)**, however, the numbers of effective flax molecular markers were still limited, and some marker types (RAPD, RFLP and AFLP) are not easily reproducible or quite laborious. The microsatellites, also known as simple sequence repeats (SSRs), are considered as ideal markers due to high polymorphic, codominant and comparatively simple and inexpensive production **(Powell *et al*., 1996)** and they have been foregrounded over the last decade because the scientists found them to be remarkably versatile molecular tools **(Chambers, MacAvoy, 2005)**. The microsatellites are non-coding, mostly AT-rich DNA sequences. They also appear in the coding regions, albeit to a much lower extent and almost exclusively as a tri- and hexanucleotides, due to the deleterious effects of frame shift mutations caused by other repeat units **(Žiarovská *et al*., 2012)**. The SSRs are simple tandem repeats of short (2–10 base pairs) sequences that often vary in the number of repeats among closely related species and even between the varieties within a single species **(Powell *et al*., 1996)**. **Armour *et al*. (1999)** defined them as 2–8 bp repeats and other authors, e.g. **Goldstein and Pollock (1997)**, as 1–6 or even 1–5 bp repeats **(Schlötterer, 1998)**. The simple sequence repeats are found in a greater or lesser degree in the genomes of just about every known organism and organelle **(Chambers, MacAvoy, 2005).** The SSR markers are based on the amplification size polymorphism generated when the lines have variable numbers of these short tandem repeats in a particular locus. The abundance, distribution, reproducibility and the generally codominant nature of the SSR markers make them highly suitable for linkage mapping and genetic diversity studies **(Cloutier *et al*., 2009; Soto-Cerda *et al*., 2011; Wiesner *et al*., 2011)**. The SSR length polymorphisms at individual loci are detected by PCR using the locus specific flanking primers where the sequence is known **(Zietkiewicz *et al*., 1994)**. In the last few years, important genetic resources have been developed for flax **(Cloutier, 2012)** and the SSR markers have been successfully used for the analysis of genetic diversity **(Prassana and Pixley, 2010),** which is important for sustainable development of agricultural production.

MATERIAL AND METHODS

Plant materials and DNA extraction

The biological material of *Linum usitatissimum L.*, genotype Kyivskyi, was obtained from the experimental fields in the Chernobyl area. The DNA sample originated from the plants grown in the field situated 5 km from the Chernobyl power station. The soil is contaminated by radioactive cesium ^{137}Cs and strontium ^{90}Sr isotopes. Samples from the field on which the radioactive contaminated soil was removed and replaced with uncontaminated soil is located directly in the city of Chernobyl. The Flax DNA samples from the gene bank was used as a control. The biological material of flax in the form of extracted DNA was obtained from the Institute of Cell Biology and Genetic Engineering in Ukraine, Kiev. The genomic DNA was extracted from fresh green parts of the plant according **Rogers and Bendich (1994)**. The quantity and quality of the isolated DNA was detected by a spectrophotometer.

Amplification of microsatellite loci

The PCR reactions were based on the protocol by **Deng (2010)**. The PCR reactions were performed using the MyCycler Thermal cycle (Biorad®). The amplifications were performed by the following conditions: 95 °C for 5 minutes

followed by 35 cycles of 95℃ for 1 minute, 52℃ for 1 minute, 72℃ for 2 minutes followed by the final extension at 72°C for 10 minutes. The PCR products were separated on a 1.5 % agarose gel and visualized using GelRed Nucleic Acid Stain (Biotium®). In order to achieve the optimum reaction conditions and high specificity of amplification products, the testing and optimization of PCR was performed (temperature of binding primers, concentration of $MgCl_2$, and addition of *Taq* DNA polymerase). The temperature of binding primers (Microsynth ™) of 52℃, addition 0.5 mmol.dm^{-3} of $MgCl_2$ (Bioline ™)and 1U *Taq* DNA polymerase (Thermo Scientific ™) was selected as the most appropriate combination of conditions and components. The primer pairs according **Deng (2011)** listed in Table 1 were used for the analysis.

Table 1 SSR locus names and primer pairs sequences used for microsatellite loci

Primer name	Sequences of primers
Flax_SSR_1_for	5´-TCA TTC ATC TCC TTC CAC TAA AA-3´
Flax_SSR_1_rev	5´-TTG AAA GCC CTA GTA GAC ACC A-3´
Flax_SSR_2_for	5´-GCT CGT GAT CTC CTT CAT CC-3´
Flax_SSR_2_rev	5´-AAA ACC ACG TCC AGA TGC TC-3´
Flax_SSR_3_for	5´-CAT CCA ACA AAG GGT GGT G-3´
Flax_SSR_3_rev	5´-GGA ACA AAG GGT AGC CAT GA-3´
Flax_SSR_4_for	5´-TCC CGT AAT ATT CTA TGT TCT TCC-3´
Flax_SSR_4_rev	5´-TGA GTT GGA CCT TAC AAG ACT CA-3´
Flax_SSR_5_for	5´-AAG GGT GGT GGT GGG AAC-3´
Flax_SSR_5_rev	5´-GTT GGG GTG AAG AGG AAC AA-3´
Flax_SSR_6_for	5´-ATG GCA GGT TCT GCT GTT TC-3´
Flax_SSR_6_rev	5´-TTG CGT GAT TAT CTG CTT CG-3´
Flax_SSR_7_for	5´-TCT ACA GAG TCC AAT TCC CGT AA-3´
Flax_SSR_7_rev	5´-GTT GGA CCT TAC AAG ACT CAC TG-3´
Flax_SSR_8_for	5´-GTT TGA GAA GAG GGC ATC CA-3´
Flax_SSR_8_rev	5´-GGT GGG GTG AAG AGG AA-3´

Legend: SSR – simple sequence repeats, for – forward, rev – reverse, A – adenine, T – timine, C – cytosine, G – guanine

RESULTS

The molecular marker techniques have only been used in flax gene pool analysis in the recent past. The first studies dealing with the genetic diversity of this species used the isoenzymes as markers. The DNA analysis offers another possibility for the exploration of different plant species at the molecular level. Nowadays, there are several techniques for mapping the polymorphism of individual DNA regions, which use specifically designed primers **(Žiarovská *et al*., 2012)**.
The microsatellite loci were amplified by the SSR method using eight different primer pairs. The amplification conditions have been optimized due to the presence of additional components of the flax DNA. It is necessary to add that the SSR experiments were carried out using the DNA transported from the original site. The PCR condition has been optimized as follows: the annealing temperature of primers was adapted by a gradient PCR, concentration of magnesium and *Taq* polymerase. The optimum annealing temperature was set at 52 ℃. The results are shown in Fig. 1.

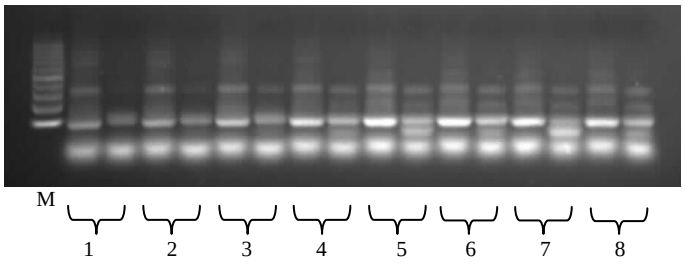

Figure 1 PCR electropherogram – temperature gradient for primer flax_SSR_1
M – molecular marker, temperature optimization of PCR: 1 - 50°C, 2 – 50,7 °C, 3 - 52°C, 4 – 53,9°C, 5 – 56,3°C, 6 – 58,3°C, 7 – 59,4°C, 8 - 60°C

However, the tested temperature incensement did not have any effect on the product amplification. The quantity of $MgCl_2$ was adjusted in the second step of optimization. The Dream *Taq* Master Mix contains 4mmol.dm^{-}3 of $MgCl_2$. This concentration was increased by adding an additional amount of $MgCl_2$ in the concentration of 0.5 mmol.dm^{-3} of $MgCl_2$ (Figure 2).

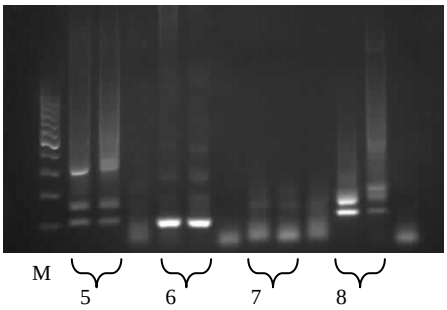

Figure 2 PCR electropherogram – increased concentration of $MgCl_2$ in the reaction mixture.
M – molecular marker, 5 – flax_SSR_5, 6 – flax_SSR_6, 7 – flax_SSR_7, 8 – flax_SSR_8

In the last step of optimization, 1U of polymerase was added to each individual reaction. The PCR products were clearly visible (Figure 3).

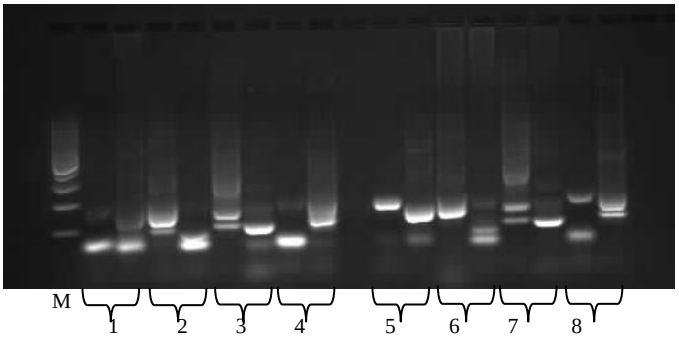

Figure 3 PCR electropherogram – addition of 1U polymerase
M – molecular marker, 1 - lan_SSR_1, 2 - lan_SSR_2, 3 - lan_SSR_3, 4 - lan_SSR_4, 5 - lan_SSR_5, 6 - lan_SSR_6, 7 - lan_SSR_7, 8 - lan_SSR_8

Using eight pairs of microsatellite primers, we successfully amplified the samples from the remediated fields. For each primer in the control samples and remediated samples, we detected 1 to 3 fragments per locus, each in size up to 120 to 250 base pairs. The applied microsatellite primers confirmed the monomorphic condition of the microsatellite loci (Table 2).

Table 2 Comparison of the number and sizes of alleles

Primer pairs	Detected in this study		Results by Deng (2011)	
	Number of alleles	Size (bp)	Number of alleles	Size (bp)
Flax_SSR_1	2	90, 170	4	146-179
Flax_SSR_2	1	150	4	153-162
Flax_SSR_3	1	160	5	134-146
Flax_SSR_4	-	-	12	144-228
Flax_SSR_5	3	120, 170, 250	4	97-147
Flax_SSR_6	1	130	8	90-138
Flax_SSR_7	1	190	6	153-294
Flax_SSR_8	2	160, 180	4	158-167

Legend: SSR – simple sequence repeats, bp – base pairs

DISCUSSION

The study of impact of environmental change on species and ecosystems is a vital area of research. This includes the study of adaptation of organisms to unfavorable environmental conditions and survival mechanisms (**Voronova et al., 2011**). Especially long-term mutagenic impact of low-dose-rate chronic has become increasingly topical (**Karimullina et al., 2013**) because a new concept of radiation protection of humans and biota must be developed on the basis of a clear understanding of the regularities of the formation of the biological effects of low-dose ionizing radiation (**Geraskin, 2013**). Plants provide a wonderful opportunity for the study of genome variation.
The base of the reproducible analysis for the methodologies of genetic technologies is the isolation and purity of the total isolated genomic DNA. For analyses, the genomic DNA of flax was extracted from fresh leaves according to **Rogers and Bendich (1994)**. The analysis of polymorphism of the existing microsatellites loci in the flax genome from the Chernobyl area was done by eight primer pairs, the sequences of which were published by **Deng et al. (2011)**. In total, 12 fragments were amplified by the primers (1-3 fragments for each primer). The size of fragments varied from 90 to 250 bp. **Deng et al. (2011)**

obtained 37 fragments (4-12 alleles per locus) by the same primers. The sizes of alleles were from 90 to 228 bp.

By using 23 genomic SSRs and an analysis of 93 flax cultivars, **Roose-Amsaleg *et al.* (2006)** obtained 93 alleles varying from 2 to 8 (average of 3.32) alleles per locus. Similarly, on the bases of 35 genomic SSRs from 8 cultivars, **Deng *et al.* (2010)** obtained 113 alleles varying from 2-6 (average of 3.45) alleles per locus. With a microsatellite analysis using 54 primes, **Soto-Cerda *et al.* (2011)** amplified 180 alleles, 2-10 (average of 3) alleles per locus. **Rachinskaya *et al.* (2011)** analyzed 15 cultivars of flax by 20 primers pairs, obtaining 67 alleles, 2-7 (average of 3.05) alleles per one locus. The trinucleotide microsatellite motives are most frequently in the plant genome. This was confirmed by several authors in different plant species: wheat **(Song *et al*., 2002)**, peas **(Burstin *et al*., 2001)**, chickpeas **(Choudhary *et al*., 2009)**, barley **(Thiel *et al*., 2003),** flax **(Cloutier *et al*., 2009)**, cotton **(Guo *et al*., 2007)**. **Cloutier *et al*. (2009)** found that the trinucleotide TTC motive was the most abundant in the flax EST-SSR. **Lifeng *et al*. (2003)** analyzed the microsatellites in four main crops by using EST – SSRs: wheat, rice, corn, soybeans and detected that the TTC motive is the most represented in the rice and soybeans genomes. By discovering 37 new SSRs primers derived from the EST library, **Bassil *et al*. (2006)** pointed that the TTC motive was one of the most trinucleotide repeat in strawberries (38%). The TTC motive was represented at 48% in the genome of the model plant of *Arabidopsis thaliana* **(Cardle *et. al*., 2000).**

CONCLUSION

The plants in the Chernobyl area had to adapt to ionizing radiation and the molecular base of their adaptive reactions is of a high interest to the researches. The molecular characterization of living organisms, especially plants, in the radioactive areas could allow for agricultural use of these areas and plants in the future.

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