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ASSESSING THE MOST APPROPRIATE PCR CONDITIONS FOR SSR MARKERS IN WHEAT CULTIVARS STUDY

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Abstract

Although it is well known that molecular biology techniques have recently seen a significant increase in the development of new research methods, an important role when choosing the appropriate working methods lies not only in the methods acceptance criteria such as specificity, applicability, robustness but also in the final cost of the implemented technology. Therefore, although methods involving SSR markers are much more specific and informative than other and these markers can be easily identified by PCR techniques, difficulties may arise in interpreting the results if the chosen SSR markers are not suitable for researches purposes or the conditions for PCR reaction are not appropriate. The methods optimization plays an important role in obtaining the desired results.
The aim of this study was to choose the most suitable PCR conditions optimizing either the reaction parameters by varying the reagents used or their concentration as well as PCR amplification conditions when using fourteen SSRs markers in wheat cultivars grown in Romania. The SSRs markers DuPw167, DuPw217, DuPw004, DuPw115, DuPw205, Xgwm155, Xgwm413, Xgwm003, Xgwm372, Xbarc184, Xbarc347, Xbarc074, Xgwm052 and Xgwm095 were amplified with three PCR reaction mixtures at different annealing temperatures. All SSRs markers gave the best amplification profiles with the PCR mix 2 which had as reagent a Hot Start Taq DNA Polymerase.
By choosing the best methods conditions a successful amplification and a unique allele profile may be provided thus being able to standardize the methods used.

Key words*: SSR markers, PCR conditions, methods optimization, wheat.*

INTRODUCTION

Wheat is one of the main cereal crops and if we refer to the evolution of agricultural researches in this field, we can mention the fact that molecular biology methods and especially those using molecular markers or PCR based technology have provided in the last decades new opportunities for researchers (Yadav et al., 2019; Kumar et al., 2009).
Molecular markers have many applications such as marker assisted selection (MAS), being used in breeding programs, genetic purity assessment and variety identification, quantitative trait loci mapping (QTLs) (Bernardo, 2008; Raza et al., 2019; Nadeem et al., 2018; Noli et al., 2008; Niaz et al., 2020). Thus, using molecular markers in molecular biology techniques plays an important part for researchers and nowadays there are numerous research studies that designate Simple Sequence Repeats or SSRs markers as being among the most used molecular markers in wheat study and not only (Varshney et al., 2000; Hayden et al., 2008; Haque et al., 2021).
SSRs or microsatellite markers are valuable tools for studying plant genetic material. These markers are represented by sequences of 1-6 nucleotides repeated in tandem. SSR markers are found in abundance in the plant genome but may also exist in other places, such as mitochondria (mtSSRs) or chloroplast (cpSSRs) (Nadeem et al., 2018). Due to the high level of polymorphism, they can be easily identified by PCR technique (Raza et al., 2019; Elameen & Mohamed, 2019; Erayman et al., 2016).

When it comes to molecular biology methods used for wheat we can remark that the methods involving SSRs are widely used. Among the reasons for their excessive use we can list that SSRs markers are more informative than other markers with important implications in breading program where marker assisted selection speeds up the process (Madesis et al., 2013; Xu & Crouch, 2008; Brumlop & Finckh, 2010; Ciucă et al., 2018; Cristina et al., 2018; Nadeem et al., 2018; Dinu et al., 2014) in genetic diversity assessment (Zhao et al., 2019; El-Fiki & Adly, 2019; Mangini et al., 2010; Xinquan et al., 2005) and genotyping the wheat genomes (Röder et al., 1998; Eujayl et al., 2002).

When choosing the most appropriate method

Several aspects should be taken into account when choosing the working method. Thus, the methods evaluation regarding applicability, practicability, robustness, reproducibility plays an important role in its selection and using SSRs markers has advantages in terms of method specificity.

SSRs markers are codominant markers that have a high level of polymorphism being easily identified by PCR technique (Raza et al., 2019).

A first important factor for the selected method to work within the chosen limits is to select molecular markers that can highlight the genetic polymorphism associated with the biological material used. The allelic profiles resulting from amplification as well as the polymorphism associated with the use of markers play an important role in choosing the right method (Khan et al., 2014). Although SSRs markers are much more specific and informative than other markers, difficulties may arise in interpreting the results if the chosen markers are not suitable for the purpose (Senan et al., 2014; Sun et al., 2020). Thus, the selected methods regardless the purpose should use reliable markers so that the results can be quickly obtained.

The technology should be easy to use and not to generate high costs and should not require a large amount of DNA or prior information about the plant genome.

As advantages for development of methods based on the use of SSRs markers are that these methods use a small amount of DNA that does not require high purification, and the results can be obtained much faster (Raza et al., 2019, Nadeem et al., 2018).

This study aimed to find the best working conditions for fourteen SSRs markers recommended in wheat varietal purity assessment (ISTA, 2021).

MATERIALS AND METHODS

Selection of samples material and DNA extraction

Plant material consisted of certified wheat seeds obtained from Central Laboratory for Quality of Seeds and Planting Material (LCCSMS), Romania.

DNA extraction

In this study DNA extraction was performed using NucleoSpin Plant II extraction kit (Macherey-Nagel) and the protocol was performed according to the manufacturer's requirements and adapted to meet the desired concentration and purity requirements (Macherey-Nagel, 2019).

DNA was extracted from dry wheat seeds as follows: from each homogenized seed samples a number of 3-4 seeds were ground with a mortar and pestle and approximately 40 mg of ground powder was transferred to a sterile 2 ml microcentrifuge tube.

The extraction kit has included two lysis buffers based on CTAB and SDS methods. In this study the DNA was extracted with CTAB based buffer. The mixture was vortexed thoroughly and RNase A solution has been added to the mixture. The suspension was incubated for 30 min at 65°C. The lysates were cleared by centrifugation and/ or filtration using the kit column in order to remove polysaccharides, contaminations and/or other residual cellular debris and mixed with kit binding buffer. After loading this mixture on a silica membrane spin column, the contaminants were washed three times using kit wash buffers. The genomic DNA was eluted with a low salt elution buffer (5 mM Tris/HCl, pH 8.5). After extraction the genomic DNA extracted can be kept at 4°C for a short period

of time for further analysis but should be frozen at -20 °C for longer storage. (Macherey - Nagel, 2019).

Genomic DNA evaluation

The DNA concentration and quality were evaluated by spectrophotometry using Biochrom Biowave DNA UV-Vis Spectrophotometer.

SSRs markers

Wheat SSRs markers for this study were chosen according to International Seed Testing Association (ISTA) rules being recommended as suitable for verification of wheat varieties (ISTA, 2021). In Table 1 the SSRs markers and PCR primers sequence used for optimizing the methods in this study is presented.

Table 1. PCR primers sequence of the SSRs markers used in this study

SSRs marker	Forward	Reverse
DuPw167	CGGAGCAAGGACGATAGG	CACCACACCAATCAGGAACC
DuPw217	CGAATTACACTTCCTTCTTCCG	CGAGCGTGTCTAACAAGTGC
DuPw004	GGTCTGGTCGGAGAAGAAGC	TGGGAGCGTACGTTGTATCC
DuPw115	TGTTTCTTCCTCGCGTAACC	CCTCGAATCTCCCAGTTATCG
DuPw205	ATCCAGATCACACCAAACGG	CTTCCGCTTCATCTTCTTGC
Xgwm155	CAATCATTTCCCCCTCCC	AATCATTGGAAATCCATATGCC
Xgwm413	TGCTTGTCTAGATTGCTTGGG	GATCGTCTCGTCCTTGGCA
Xgwm003	GCAGCGGCACTGGTACATTT	AATATCGCATCACTATCCCA
Xgwm372	AATAGAGCCCTGGGACTGGG	GAAGGACGACATTCCACCTG
Xbarc347	GCGCACCTCTCCTCACCTTCT	GCGAACATGGAAATGAAAACTATCT
Xbarc184	TTCGGTGATATCTTTTCCCCTTGA	CCGAGTTGACTGTGTGGGCTTGCTG
Xbarc074	GCGCTTGCCCCTTCAGGCGAG	CGCGGGAGAACCACCAGTGACAGAGC
Xgwm052	CTATGAGGCGGAGGTTGAAG	TGCGGTGCTCTTCCATTT
Xgwm095	GATCAAACACACACCCCTCC	AATGCAAAGTGAAAAACCCG

(ISTA, 2021; Eujayl et al., 2002; Röder et al., 1998; Song et al., 2005)

Choosing PCR conditions

To choose the best PCR conditions was made an experimental plan that consisted of evaluating PCR products resulting from the PCR amplification process with the chosen SSRs markers. For this purpose, three PCR reaction mixtures were made.

Mixture one containing PCR mix composed of 1x concentration Fast Start PCR Master, a ready-to-use, double-concentrated hot start PCR mix (Roche) which contains a Fast Start Taq DNA Polymerase, nucleotides and all other reagents required for PCR reaction.

Mixture two containing PCR mix composed of 1 x concentration from 5 x Green GoTaq Flexi Buffer, 1.5 mM $MgCl_2$ solution 25 mM, 0.2 mM PCR Nucleotide Mix 10 mM, 1.25 u GoTaq Hot Start Polymerase (5 u/µL) (Promega).

Mixture three containing PCR mix composed of 1 x DreamTaq Buffer which includes 20 mM $MgCl_2$, 0.2 mM PCR Nucleotide Mix 10 mM, 1.25 u Thermo Scientific DreamTaq DNA Polymerase.

Additional reagents for all mixture: nuclease-free water up to 15-50 µL final volume, upstream and downstream primer at a final concentration of 0.3 µM and 30-40 ng/µL template DNA. Table 2 presents the final concentrations for all reagents contained in the mentioned mixtures.

Table 2. PCR component reaction and final concentration

PCR Mix	PCR component reaction	Final Concentration
1	FastStart PCR Master 2x	1 x
	upstream primer	0.3 µM
	downstream primer	0.3 µM
	template DNA	5 µL
	Nuclease-Free Water to	50 µL
2	5 x Green GoTaq® Flexi Buffer	1 x
	MgCl2 Solution, 25 mM	1.5 mM
	PCR Nucleotide Mix, 10 mM each	0.2 mM
	upstream primer	0.3 µM
	downstream primer	0.3 µM
	GoTaq® Hot Start Polymerase (5u/µl)	1.25 u

PCR Mix	PCR component reaction	Final Concentration
	template DNA	3 µL
	Nuclease-Free Water to	15 µL
3	10X DreamTaq Buffer	1 x
	PCR Nucleotide Mix, 10mM each	0.2 mM
	upstream primer	0.3 µM
	downstream primer	0.3 µM
	DreamTaq DNA Polymerase	1.25 u
	template DNA	3 µL
	Nuclease-Free Water to	20 µL

For PCR mix 3, additional optimization was performed and variations consisted in primers final concentration within a range of 0.2 to 0.4 µM, additional amount of 25 mM $MgCl_2$ added to the PCR mix and also in mix final volume from 15 to 20 µL were made.

Thermal cycling profile for PCR amplification for chosen SSRs markers was also evaluated.

In Table 3 is presented the thermal cycling profile for PCR amplification, variations made on number of cycles, annealing temperature range and cycles duration. PCR amplification was performed in 96 well BIO- RAD T100™ Thermal Cycler system.

Table 3. Thermal cycling profile for PCR amplification and its variation

Number of cycles	Program	Temperature	Duration
1	Initial Denaturation	95°C	3- 5 min
34-45	Denaturation	95°C	30 s-1 min
	Annealing	48-65°C	30 s-1 min
	Extension	72°C	30 s-1 min
1	Final extension	72°C	5 min
1	Hold	12°C	indefinitely

Fragment separation and detection for PCR products resulted after amplification was done by agarose gel electrophoresis. PCR products were analysed using an agarose gel with a concentration in range of 2.3-2.6% agarose (Agarose I TM/VWR and Agarose, LE, Analytical Grade/Promega) in 1 x TAE buffer (Rothiphorese 10 x TAE Buffer from Roth).

For detecting nucleic acid in agarose gel was used Red Safe™ Nucleic Acid Staining Solution (Intron) a substitute for EtBr (ethidium bromide).

Electrophoresis power supply was insured by Consort EV243 and the migration was performed at a voltage between 66-90 V. The migration time was between 45 minutes and two hours. PCR products were visualized on UV light using Vilber Lourmat E - Box VX2 imaging analysis system.

RESULTS AND DISCUSSIONS

An important step when talking about molecular biology techniques that uses PCR technology is DNA extraction. The chosen extraction method must lead to a template DNA that meets methods quality and quantity requirements and when using SSRs markers the DNA requirements are small amount of template DNA that does not require high purification (Raza et al., 2019; Nadeem et al., 2018)

As previously mentioned, DNA extraction in this study was performed using an extraction kit. The manufacturer successfully tested both lysis buffers (CTAB and SDS based lysis buffer) on wheat leaves (Macherey-Nagel, 2019). In this study only CTAB lysis buffer was tested on dry seeds and the chosen buffer gave the expected results for extracted DNA.

The DNA yield was between 60-100 ng/µL and the average of the measurements for the purity ratio was 1.8 for A_{260}/A_{280} ratio and 2 for A_{260}/A_{230} ratio. The extracted genomic DNA, thus had all the qualities regarding the purity ratio and the desired concentration to go further with PCR amplification. Seeing that other studies show that an SDS-based lysis buffer is more suitable for DNA extraction from wheat dry seeds (Cristina et al., 2017) this buffer is also considered for testing in future studies.

As chosen method must meet the conditions in terms of its specificity and reproducibility (Agarwal et al., 2008) when validating a method, it is important to meet all the acceptability criteria (Wood, 1999) so it becomes necessary to standardize the technique involving the use of SSRs and to choose for this purpose the most informative markers.

All SSRs markers chosen in this study gave good amplification products

Another important stage in order to optimize the working methods is to choose the right PCR conditions for amplifying SSRs markers.

The experimental plan consisted in choosing for each SSRs marker the reaction mixture and the most suitable annealing temperature so that the resulting amplification products can be well highlighted without non-specific amplification products, thus allowing a good results interpretation.

All markers were tested under reaction conditions in which PCR reaction mix, the primers annealing temperature as well as the number of amplification cycles varied. For each SSR marker, several combinations of PCR reaction mixtures and annealing temperatures were tested.

The variations regarding the PCR reaction mixtures, annealing temperatures, number of amplification cycles used for each SSR marker but also details and remarks regarding the PCR products obtained after amplification under the given conditions can be observed in Table 4.

Table 4. Variation data on protocols and results obtained for each SSRs marker under present study

SSRs markers	PCR Mix	Annealing temperature/ gradient (°C)	Number of cycles	Amplification products			Remarks
				mix 1	mix 2	mix 3	
DuPw167	1	57.6 ;56.1; 54; 53.3	35	+;+; +;+	No	No	- good products amplification for all temp with mix 1; - chosen temperature 54°C for mix 1 and 2; - with mix 2 the products are much well highlighted than with mix 1 and 3; - mix 3 needs more optimization.
	1& 2	54	35	+	+	No	
	2 & 3	54	40	No	+	+, Ns.p.	
DuPw217	1 & 2	61.0; 56.1; 54.5; 53.0	35	-; +; +;+	+; +; +; +	No	- no products amplification with mix 1 at 61°C; - with mix 2 the products are much well highlighted - chosen temperature for this marker 55°C - optimization for mix 3 is needed (more non-specific amplification products).
	2	55	35	No	+	No	
	2 & 3	55	40	No	+	+, Ns.p.	
DuPw004	1 & 2	55; 54; 51	35	+; +; +	+; +, Ns.p; +, Ns.p	No	- chosen temperature 55°C for all PCR mix; - with mix 2 the products are highlighted much well at all temperatures; - non-specific amplifications products with mix 2 and 3; - the results are reproducible; - between 65°C - 55.3°C good amplification at all temp for mix 1 & 2.
	2	65	35	No	+	No	
	2	54	35	No	+, Ns.p	No	
	1 & 2	54	35	+	+, Ns.p	No	
	1 & 2	65;60.3;55.3	35	+; +; +	+; +; +	No	
	2 & 3	55	40	No	+	+, Ns.p.	
DuPw115	1 & 2	55; 54;51°C	35	+; +; +	+; +; +	No	- with mix 2 the products are much well highlighted for all temperatures; - no amplification products at 65°C - chosen temperature 56°C for mix 2 - poor amplification with mix 1 (needs optimization); - non-specific amplifications products with mix 3
	2	54	35	No	+	No	
	2	65; 60.3;55.3	35	No	-; +; +	No	
	1 & 2	56	35	No	+	No	
	2 & 3	56	40	No	+	+; Ns.p.	
DuPw205	1 & 2	55; 54; 51	35	+; +; +	+; +; +	No	- with PCR mix 1 & 2 between 55°C- 51°C the products are not well highlighted, reaction need optimization; - the results are reproducible; - chosen temperature 60°C for mix 2 - results reproducibility and repeatability when chosen temperature is 60° (for PCR mix 2 variables in number of cycles from 34 to 40 and variation on the use of another hot start enzyme GoTaq G2 Polymerase) and for PCR Mix 1 the use of Fast start master 480 probes master (Roche) when they were not non-specific amplifications products; - non-specific amplifications products with PCR mix 3 at chosen temperature.
	2	54	35	No	+	No	
	2	60; 58.8; 57.7; 56.3	34	No	+; +; +; +	No	
	2	60	40	No	+	No	
	2	60; 58.2 ;56.5; 51	35	No	+; +; +; +	No	
	2 & 3	60	40	No	+	+, Ns.p.	
Xgwm155	1 & 2	54; 52.8; 48	35	+; +; +	+, Ns.p; +, Ns.p; +, Ns.p	No	- between 54°C-48°C non-specific amplifications with mix 2, with mix 1 the products are not well highlighted, reaction need optimization - 60°C with PCR mix 1 the products are not well highlighted -62.4°C-61.4°C no amplification with PCR mix 2; - chosen temperature 56°C for PCR mix 2; - non- specific amplifications products with PCR mix 3.
	1 & 2	60	45	+	+	No	
	2	62.4; 61.4; 59.9; 55.6	40	No	-; -; +; +	No	
	2 & 3	56	40	No	+	+, Ns.p.	
Xgwm413	1 & 2	54; 52.8;48	35	+; +; +	+, Ns.p; +, Ns.p; +, Ns.p	No	- 54°C-48°C non-specific amplifications with mix 2 and with mix 1 the products are not well highlighted, the reaction needs more optimization - chosen temperature 60°C for PCR mix 2 with PCR mix 1 the products are not well highlighted - results are confirmed at chosen temperature - non-specific amplifications products with PCR mix 3
	1 & 2	60	45	+	+	No	
	2	62.4;61.4; 59.9; 55.6	40	No	+; +; +; +	No	
	2	60	40	No	+	No	
	2 & 3	60	40	No	+	+, Ns.p.	
Xgwm003	1 & 2	54; 52.8; 48	35	+; +; +	+; +, Ns.p; +, Ns.p	No	- 54°C-48°C with PCR mix 1 the products are not well highlighted, optimizing the reaction is needed/ non-specific amplification products with PCR mix 2;
	1 & 2	55.2	45	+	+	No	

SSRs markers	PCR Mix	Annealing temperature/ gradient (°C)	Number of cycles	Amplification products			Remarks
				mix 1	mix 2	mix 3	
	2	59.9; 58.1; 56.5; 55	40	No	+; +; +; +	No	- chosen temperature 55°C for PCR mix 2; - non-specific amplifications products with PCR mix 3.
	2 & 3	55	40	No	+	+, Ns.p.	
Xgwm372	1 & 2	60	35	+	+	No	- at 60°C with PCR mix 1 the products are not well highlighted - non-specific amplifications products with PCR mix 3, the reaction needs more optimization - variation in primers final concentration with PCR mix 3 are made (0.2 µM primers final concentration); - chosen temperature 61°C for PCR mix 2 & 3
	3	60	40	No	No	+, Ns.p.	
	3	61	40	No	No	+	
	3	61.4 ;59.9; 58.1; 56.5	40	No	No	+; +; +; +	
	2 & 3	61	40	No	+	+	
Xbarc347	3	61; 60.5;58.7; 57.3; 55.3	40	No	No	+, Ns.p.; +, Ns.p.; +, Ns.p.; +, Ns.p.; +, Ns.p.	- more non-specific amplification products with this marker when using PCR mix 3 than PCR mix 2 -variation in primers final concentration with PCR mix 3 are made (from 0.2 to 0.4 µM primers final concentration); - chosen temp 57°C with PCR mix 2
	3	57	40	No	No	+, Ns.p.	
	3 & 2	58; 57.4; 56.1; 55.1	40	No	+; +; +; +	+, Ns.p.; +, Ns.p.; +, Ns.p.; +, Ns.p.	
	2	57	40	No	+	No	
Xbarc184	1 & 2	58	35	+	+	No	- with PCR mix 1 the products are not well highlighted - results are confirmed for PCR mix 2 at chosen temperature - chosen temp 58°C with PCR mix 2 and all PCR products are well highlighted at all temperatures (good amplification products with PCR mix 2 & 3- with PCR mix 1 the products are not well highlighted
	2	58	40	No	+	No	
	2	58; 57.4; 56.1; 55.1	40	No	+; +; +; +	No	
	3 & 2	58	40	No	+	+	
Xbarc074	1 & 2	60	35	+	+	No	- at 60°C with PCR mix 1 the products are not well highlighted - good amplification between 60°C-58°C for PCR mix 2, non-specific products amplification at 56.1°C - chosen temp 60°C for PCR mix 2 with this marker, - repetitive results at chosen temperature
	2	61; 60.5; 58.7; 56.1	40	No	+; +; +; +, Ns.p	No	
	2	60	40	No	+	No	
Xgwm052	2	61.2; 60; 58.8	40	No	+; +; +	No	- good amplification for PCR mix 2 at all temperatures, - working temperature chosen for this marker is 60°C for PCR mix 2; repetitive results at chosen temperature
	2	60	40	No	+	No	
Xgwm095	1 & 2	60	35	+	+	No	- with PCR mix 1 the products are not well highlighted - good amplification products for PCR mix 2 at all temperatures, the chosen temperature for this marker is 60°C for PCR mix 2, repetitive results at chosen temperature with PCR mix 2
	2	61.2; 60; 58.8	40	No	+; +; +	No	
	2	60	40	No	+	No	

Legend: "+"- presence of amplification products, "-" - absence of amplification products; "Ns.p."- Non-specific amplification products, "No"- no amplification in these conditions was done.

Following the data analysis from Table 4 we can say in terms of using the appropriate PCR reactions mix that the best results were obtained for all SSRs markers included in the study by using the PCR reaction mix 2.

The analysis of obtained results with the three PCR reaction mixtures showed that the PCR products resulting from amplification with PCR mix 2 were better highlighted than when PCR mix 1was used, so in Figure 1 can be observed the products resulting from the amplification with DuPw167 marker of four wheat varieties with PCR mix 2 and PCR mix 1 at the chosen annealing temperatures 54°C.

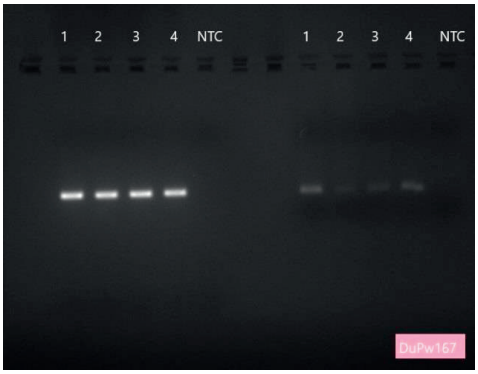

Figure 1. Agarose gel electrophoresis of PCR product obtained with DuPw167 marker

Legend: "1-4" - wheat varieties tested, on the right are products obtained with PCR mix 1 and on the left products obtained with PCR mix 2, NTC- negative control which does not contain template DNA.

Even if with mix 1 the amplification products are not well highlighted compared to mix 2 and 3, non-specific products could not be observed in this study under the tested conditions. Another observation regarding the use of PCR mix 1 is that depending on the molecular markers and the annealing temperature used, the products are either not very well highlighted and at certain temperatures products cannot be observed. Thus, for DuPw217 marker no amplification products can be observed with PCR mix 1 at the annealing temperature of 61°C while with mix 2 the products are present. Since the annealing temperature chosen for future PCR reactions with this marker is 55°C, the absence of PCR products at 61°C with PCR mix 1 is not of great significance for the present study.

The same was observed for the DuPw115 marker when it is not relevant that at annealing temperature of 65°C no amplification products with PCR mix 2 are obtained because the temperature chosen for future research with this marker is 56°C.

Following the data analysis, it was found that variations related to the use of another Taq DNA polymerase namely GoTaq G_2 Hot Start DNA Polymerase (Promega) for PCR mix two or the use of a new ready-to-use mix (Fast start master 480 probes master from Roche) instead of mix 1 did not influence the amplification result at the annealing temperature of 60°C chosen for DuPw205 marker.

Regarding the PCR mix 3, it was observed that there are many more non-specific amplifications products than when the PCR mix 2 is used. The presence of non-specific amplification products when working with mix 3 compared to mix 2 can be observed in Figure 2. Thus, when using the Xbarc347 marker, it could be observed that under the conditions of the present study and at the chosen temperatures there are more non-specific products when using mix 3 than when using mix 2. This can be explained by the fact that the enzyme GoTaq® Hot Start Polymerase used in mix 2 has higher reaction specificity than DreamTaq DNA Polymerase from mix 3.

In Figure 2 can be observed the products resulting from the amplification with Xbarc347 marker of two wheat varieties with PCR mix 3 and PCR mix 2 at annealing temperatures of 58°C, 57.4°C, 56.1°C and 55.1°C.

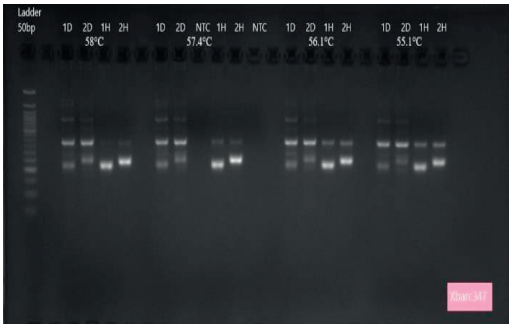

Figure 2. Agarose gel electrophoresis of PCR product obtained with Xbarc347 marker
Legend: "1, 2" - two of the wheat varieties tested; "D" - amplification with PCR mix 3; "H" - amplification with PCR mix 2; NTC - negative control which does not contain template DNA

In order to reduce the presence of non-specific amplification products that appeared under the tested conditions in some of the SSR markers it was decided that for PCR mix 3 variations should be made.

For SSR markers Xgwm372 and Xbarc347 with PCR mix 3 variations in primers final concentration (from 0.2-0.4 µM), additional 25 mM $MgCl_2$ added to the master mix and reaction final volume were made. No significant differences were reported under tested conditions and it was decided that the values chosen in Table 2 are appropriate. This fact suggests that for the use of PCR mix 3 in future studies a better optimization for PCR reaction conditions is needed in case of some SSRs markers.

The starting point in order to select the right annealing temperature for the selected SSRs markers under the conditions of the present study was to perform a gradient temperature either starting from primers melting temperatures (Tm) or from selected annealing temperature from online resources or literature (Blake et al., 2019; El-Fiki & Adly, 2019; Eujayl et al., 2002; Röder et al., 1998; Song et al., 2005; ISTA, 2021). So, for a better optimization a gradient temperature around these values in accordance with PCR equipment capacity was achieved.

Annealing temperature for all SSR markers recommended by ISTA (2021) is 58°C but it

must be taken into account that a multiplex reaction was performed (ISTA, 2021).
In Figure 3 agarose gel electrophoresis of PCR product obtained with Xgwm155 using PCR mix 2 is presented. For Xgwm155 marker the annealing temperature from literature is 60°C (Blake et al., 2019) but as can be seen in Figure 3 the products are better highlighted with PCR mix 2 at a temperature of 55.6°C and therefore for this marker annealing temperature of 56°C when working with PCR mix 2 was chosen.

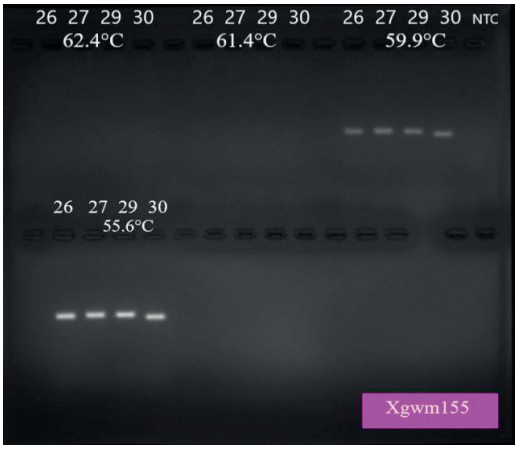

Figure 3. Agarose gel electrophoresis of PCR product obtained with Xgwm155 from four wheat cultivars using mix 2

The final annealing temperature chosen for the SSR markers in the present study conditions with the three PCR reaction mixtures was: 54°C for DuPw167 marker, 55°C for DuPw217, DuPw004 and Xgwm003, 56°C for DuPw115 and Xgwm155, 57°C for Xbarc347, 58°C for Xbarc184, 60°C for DuPw205, Xgwm413, Xbarc074, Xgwm052 and Xgwm095 and 61°C for Xgwm372.
At the chosen temperatures even if all the PCR reaction mixtures tested gave amplification the best results were obtained with mix 2.
Starting from the observations made in Table 4 a common annealing temperature can be chosen for all SSR markers with the three PCR mixtures tested in the present study that would allow in future studies the realization of a multiplex PCR reaction.
If we refer to the thermal cycling profile and the variation included in Table 3 (increase in number of cycles or their duration) no significant differences were observed.
As can be seen in Table 4 for all SSR markers included in the present study, the PCR reaction was repeated at the chosen annealing temperature with some of the PCR mixtures and all results were reproducible under the tested conditions.
The present methodology chosen for SSR markers in wheat cultivars study proved that the requirements regarding the specificity and robustness of the method are met thus methods remaining unaffected by small deviation from experimental condition.
Although the evolution of molecular biology techniques is constantly playing an important role in choosing the working method and given the rise of modern technologies such as sequencing techniques (NGS) which has made it possible to detect SNPs with a much faster yield (Zhang et al., 2020; Sun et al., 2020) there is also a problem with the final cost of the implemented technology and all these new technologies may be not yet fully accessible.

CONCLUSIONS

The chosen DNA isolation method for wheat cultivars gave in this study good amplification results.
All SSRs markers gave amplification with all three PCR mixtures at the chosen annealing temperatures for each SSR markers.
From all three PCR mixtures tested in this study the use of PCR mix 2 allowed obtaining the best amplification results.
The amplification products obtained with mix 2 were better highlighted than with PCR mix 1 and 3, having also fewer non-specific amplification products compared when using PCR mix 3. Thus, of all the Taq Polymerases included in the PCR reaction mixtures, the best results were obtained with GoTaq® Hot Start Polymerase. So, even if the use of a Hot Start Polymerase involves higher costs, the method specificity it can thus be increased.
The results remained unaffected by small variation from experimental condition.
All SSRs markers were amplified under similar test conditions with some of the PCR mixtures at the chosen annealing temperature and no

differences were reported, so results being reproducible.

Depending on the reaction mixture used especially by the enzyme included in the PCR reaction mix as well as reagents final concentration a PCR reaction optimization plan can be made for each marker, which could facilitate the method validation and method standardization.

Choosing a common annealing temperature that would allow a multiplex PCR reaction could reduce test time but it can also reduce the final costs of the analysis.

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