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PCR Protocol Optimization for Genetic Diversity Assessment and Molecular Characterization of Sour Cherry Cultivars

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Abstract. Sour cherry is an economical important tetraploid species in Europe. Use of Simple Sequence Repeats (SSRs) as descriptors to characterize and differentiate a set of 15 sour cherry varieties from three National Collections from Romania, represented by selections from local varieties and hybrids from our country and foreign origin was taken as a leading case study for plant variety protection purposes. A set of sixteen SSR markers taken from the literature which was used for genetic diversity in different genus of *Prunus* such as sweet cherry, sour cherry, peach, apricot. The PCR amplification protocol and annealing temperature for each primer pair was optimized for use in sour cherry. In these partial results, only for five (25%) primer pairs the optimum annealing temperature to amplify the sour cherry varieties was found. Similarly, 63°C and 66.2°C were found optimum for primer pairs pchgms1 and UDP96-003, only the last one from all primer pairs was polymorphic between varieties. For primer pairs pchgms2 the annealing temperature was 64°C and 66°C; 67°C and 69°C for UDP96-001; 61.3°C for UDP97-402. No more then four fragments were amplified for each varieties/primer pair combination. From these results we conclude the transferability and applicability of SSR markers for genotyping and phylogenetic studies in the genus *Prunus*.

Keywords: genetic diversity, *Prunus*, SSR markers transferability, sour cherry

INTRODUCTION

Prunus cerasus L. (sour cherry), belongs to the *Cerasus* subgenus, *Prunus* genus, and *Rosaceae* family, is a tetraploid species, with *P. avium* L. (sweet cherry, 2n = 2x = 16) and *P. fructicosa* Pall. (ground cherry, 2n = 4x = 32) proposed as its predecessor species (Beaver and Iezzoni, 1993). It is cultivated for its succulent fruit, used in consumption and industrialization process. Also, sour cherry has been used as rootstock for sweet cherry and in breeding programs for developing new commercial cultivars, dwarf and resistant rootstock (Shahi-Gharahlar, *et al.,* 2010).

The origin of *P. cerasus* was suggested by Olden and Nybom (1968), who observed a similarity between tetraploid *P. avium* and *P. fructicosa,* but the original habitat of wild sour cherry is unknown, and currently it is difficult to specify, because many wild forms have disappeared from orchards. De Candolle (1883) states the natural ranges of *P. cerasus* are overlapped around the Caspian Sea and Asia Minor, or from the northern part of Spain to the southeastern part of Russia (Hendrick, 1915). Also, Faust and Suranyi (1997), says "the sour cherries were common in Italy in antiquity" and the so called Gypsy cherries are native to the Carpathian Basin. As Handrick (1915) stated, the development of sour cherry had to happen

farther west or north from southern Russia. This can be substantiated by the location of land races. Land race can be characterized by specific descriptions under which many slightly differing clones can be grouped (Iezzoni *et al.* 1992).

Nowadays, sour cherry still grows wild in various parts of Europe, from Scandinavia and the North of Turkey to the south and shows great genetic diversity (Gunars, 2010). The European germplasm of cherry consists of local forms unique to a particular place. All these local forms, which show high variation in tree and fruit size, and disease resistance, also form a majority of clonal collections (Gunars *et al.,* 2009).

In Romania the sour cherry is cultivated mainly on hills in Botosani, Iasi, Cluj, Bacau, Mures, Vaslui, Arges, Vrancea, Valcea and Dolj. There are sour cherry populations that were formed and expanded in family gardens in the plains and down to the premontane region. Cherry culture is relatively scarce in our country. In the southeastern and northwestern areas of the country there are wild populations and natural hybrids (*Prunus cerasus* x *Prunus fruticosa*), with reduced port that year after year give large productions of high quality fruit.

In the breeding program for any plants the most important and prerequisite technique is genetic variability (Khush, 2002), also, the management and conservation of genetic resources are essential for future breeding programs (Tavaud *et al.,* 2004).

Recent molecular technologies have helped to identify, characterize and manage genetic diversity in *Prunus* breeding and germplasm characterization programs. Different types of molecular markers such as isozymes, RAPD, RFLP, AFLP, SSRs and SNPs have been developed and applied (Eun Ju Cheong, 2011).

Different DNA markers were used in studies for genetic variation and differentiation in *Prunus,* such as RAPD (random amplified polymorphic DNA) (Shimada *et al.,* 2001; Cai *et al.,* 2007; Khadivi-Khub *et al.,* 2008), SSR (simple sequence repeats) (Sosinski *et al.,* 2000; Fatih Canli, 2004; Horvath *et al.,* 2008; Bouhadida *et al.,* 2009; Nas *et al.,* 2011), AFLP (amplified fragment length polymorphism) (Struss *et al.,* 2003), RFLPs (restriction fragment length polymorphism) (Kaneko *et al.,* 1986; Uematsu *et al.,* 1991), ISSR (inter-simple sequence repeats) (Liu *et al.,* 2007; Yilmaz *et al.,* 2009; Shahi-Gharahlar, *et al.,* 2011) and isozymes (Mowrey and Werner, 1990) and SNPs (Fang *et al.,* 2006).

All of the above mentioned markers have their strengths and weaknesses. Microsatellites or SSRs are arrays of short motifs, 1–6 base pairs in length. They are abundantly and uniformly distributed throughout the eukaryotic genome and codominantly inherited. Their high information content, reproducibility and easy manipulation (reviewed by Powell *et al.,* 1996) has made them the markers of choice in genetic diversity assessment, fingerprinting, and genotyping.

Benefits of SSR markers are by their uniform distribution in the genome; high polymorphism; possibility for automation of analysis; simple interpretation of the results and codominant character (Zhang *et al.,* 2008).

Romania has a very rich fruit tree germplasm fund, which includes biological material from 11 genus and species of trees and 11 genus of shrubbery and strawberry. This genetic wealth is recognized and internationally appreciated (Braniste and Butac, 2006; Braniste *et al.,* 2007).

Our objectives were to fingerprint varieties of tetraploid sour cherry from different national collections from Romania using 16 SSR primer pairs. In order for this to be achievable, the optimization of the PCR protocol, hence, the annealing temperature for each primer pair, need to be optimized.

In Romania, this is the first study on the use of SSR markers for genetic fingerprinting and determining the genetic relationships among *Prunus* subgenus *Cerasus* genotypes.

The results of the present study aimed to demonstrate the transferability of SSR markers across species belonging to the same genus and that SSR markers can be used in genetic diversity study as well as genetic identification of *Prunus cerasus* genotypes, as noted for other plant species (Roh *et al.,* 2007; Liu *et al.,* 2007; Yilmaz *et al*., 2009) or to help breeders to select diverse genotypes.

MATERIALS AND METHODS

The 15 sour cherry varieties were obtained from the three National Collections from Romania: I.C.D.P. Iasi (11 varieties), I.C.D.P. Pitesti-Maracineni (2 varieties) and S.C.D.P. Cluj-Napoca (2 varieties), represented by selections from local varieties, hybrids from Romania and other countries.

Total DNA was extracted using the protocol described by Lodhi *et al*., 1994 and modified by Pop *et al*., 2003 from young leaves which were collected in spring and then stored at -80°C. The concentration and quality of the extracted DNA was assessed using a NanoDrop ND 1000 spectrophotometer. DNA was later diluted to 50 ng/µl with nuclease-free water (Promega) for PCR amplification.

For optimization of the annealing temperature, various sets of SSR primer pairs from sweet cherry, sour cherry and peach were selected, synthesized by Generi Biotech – Czech (Tab. 1).

SSR primers used in the literature for the genus *Prunus* were developed for one species of this genus and later transferred to other species of the genus *Prunus* (Cipriani *et al.,* 1999, Downey and Iezzoni, 2000) demonstrating high intraspecific transferability within the same genus (Wunsch and Hormaza, 2002; Mnejja *et al.,* 2010; Downey and Iezzoni, 2000).

All 16 microsatellite pairs of primers were used to amplify DNA extracted from 15 *Prunus cerasus* genotypes used in this study.

PCR amplification reactions were carried out in 15 µl total volume reaction mixtures consisting of: 20 ng DNA, 0,2 uM per SSR primer (forward and reverse) and 1x PCR Master-mix (ready to use) from Lonza. For optimization different annealing temperatures were used for all 16 primer pairs.

The amplification, for optimization of the annealing temperature for the SSR primer pairs was carried out in a 96 Well Gradient Palm-Cycler CG1-96 (Corbett Research), using conventional PCR and touchdown PCR. Conventional PCR program: first cycle – denaturation was 3 min. at 94ºC, followed by 30 cycles of 45 sec. at 94ºC, 45 sec at 50-69ºC (depends on the primer) and 45 sec. at 72ºC. Touchdown PCR program: 30 cycles of 45 sec. at 94ºC, 45 sec. at 45-69ºC (depends on the primer), and 45 sec. at 72ºC. After a final incubation for 5 min at 72ºC, the samples were stored at 4ºC prior to analysis.

To evaluate the results of amplification, the PCR products were migrated on a 1.4% agarose (Sigma-Aldrich) gel in 1X TAE Buffer (242 g Tris Base (MW=121.1), 57.1 mL Glacial Acetic Acid, 100 mL 0.5 M EDTA) at 110V for 1 hours. The molecular marker used was 100bp DNA Step Ladder (Promega). Gels were visualized on a UV light Biospectrum AC Imaging System (UVP BioImaging Systems) after staining with 0.5 µg/µl Ethidium Bromide.

To detect the DNA fragments the PCR products were separated on 6% - denaturing polyacrylamide gel in 1xTBE buffer at a constant power of 550 W, 15 mA, for 90 min, after a pre-run of the gels for 30 min. The polyacrylamide electrophoresis gels were prepared as described by Benbouza *et al.* (2006). The gel solution was applied to the assembled gel plates (0.35 mm thick) using TV400-DGGE Sequencing electrophoresis apparatus (Scie-Plas). The

amplification products were denatured for 2 min at 92°C in the thermocycler and placed on ice before being applied to the gel in 2 µl containing an equal volume of stop solution.

Gel staining protocol was followed exactly as recommended by Benbouza *et al.* (2006).

Tab. 1

Sequences and source of the SSR pairs of primers used for genetic diversity in sour cherry

Crt. no.	Primer name	Primer sequences F/R	References
1.	PS12A02	GCC ACC AAT GGT TCT TCC AGC ACC AGA TGC ACC TGA	Downey and Iezzoni, 2000
2.	pchgms1	GGG TAA ATA TGC CCA TTG TGC AAT C GGA TCA TTG AAC TAC GTC AAT CCT C	Sosinki *et al.,* 2000
3.	pchgms2	GTC AAT GAG TTC AGT GTC TAC ACT C AAT CAT AAC ATC ATT CAG CCA CTG C	Sosinki *et al.,* 2000
4.	pchcms1	GTT ACA CCT CTG TCA CA CTT GGC TGG CAT TCC TA	Sosinki *et al.,* 2000
5.	pchcms2	AGG GTC GTC TCT TTG AC CTT CGT TTC AAG GCC TG	Sosinki *et al.,* 2000
6.	UDP96-001	AGT TTG ATT TTC TGA TGC ATC C TGC CAT AAG GAC CGG TAT GT	Cipriani *et al.,* 1999
7.	UDP96-003	TTG CTC AAA AGT GTC GTT GC ACA CGT AGT GCA ACA CTG GC	Cipriani *et al.,* 1999
8.	UDP97-402	TCCCATAACCAAAAAAAACACC TGGAGAAGGGTGGGTACTTG	Cipriani *et al.,* 1999
9.	UDP97-403	CTGGCTTACAACTCGCAAGC CGTCGACCAACTGAGACTCA	Cipriani *et al.,* 1999
10.	EMPA011	TGT GCT CAC TCT CTG CTG CT TGT GTG GGT TCA CAG TCT CC	Clarke and Tobutt, 2003
11.	EMPA019	TCA CTC CTG GTT CTG TGA GC CAG GGA ATA AAA ACG TCA TGG	Clarke and Tobutt, 2003
12.	PMS3	TGG ACT TCA CTC ATT TCA GAG A ACT GCA GAG AAT TTC ACA ACC A	Cantini *et al.,* 2001
13.	PMS40	TCA CTT TCG TCC ATT TTC CC TCA TTT TGG TCT TTG AGC TCG	Cantini *et al.,* 2001
14.	PceGA25	GCA ATT CGA GCT GTA TTT CAG ATG CAG TTG GCG GCT ATC ATG TCT TAC	Cantini *et al.,* 2001
15.	PceGA50	TTC CGT CCG AAG AAA TGA TTC A TAA CTA ATG CAG CAG AGC AGC ACA	Struss *et al.,* 2002
16.	PceGA59	AGA ACC AAA AGA ACG CTA AAA TC CCT AAA ATG AAC CCC TCT ACA AAT	Cantini *et al.,* 2001

RESULTS AND DISCUSSIONS

The DNA quantity obtained varied between 176 ng/µl and 1340 ng/µl and its purity varied between 2 and 2.26.

Annealing temperature is one of the most important parameters that need adjustment in the PCR. The normal range of annealing temperature is 36-75°C (Mehboob *et al.,* 2002).

Optimization of annealing temperature is an important factor for precise amplification. Annealing temperature is determined from the denaturing temperature (*Tm*). This can be calculated using different formulas, the simplest being that developed by Wallace:

$Tm = 2(A+T)+4(G+C)$

Where: *A*, *T*, *G*, *C* is the bases number of primer.

Formula gives an approximate value, but reasonably accurate for primers of 18-24 bases. The relationship between annealing temperature and denaturing temperature is one of „black boxes" of PCR reaction.

Downey and Iezzoni, 2000, used eight primers pairs, including PS12A02 primer pair to see the polymorphic SSR markers in black cherry (*Prunus serotina*). The amplification was done in gradient program and 56°C was the annealing temperature for this primer pair. The same temperature for annealing step for this primer pair, was used by J.I. Hormaza (2002), to characterize the apricot genotypes and also by Wunsch and Hormanza (2004) for genetic diversity of local Spanish sweet cherry. In all studies the results about PS12A02 primer pair were satisfactory, that produced polymorphic amplification.

In our study a selected temperature of 67°C (up to 11°C above the one recommended by the authors) for annealing did not generate non-specific products only in agarose gel, because in 6%-polyacrilamide gel the PCR product presented as a smear (Fig. 1).

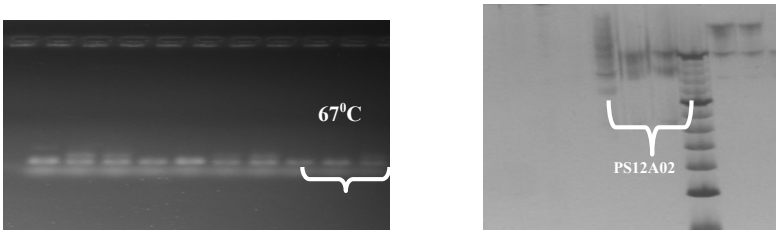

Fig 1. The PCR product obtained with the primer pair PS12A02 separated in agarose gel (A) and in 6%-polyacrilamide gel (B)

The group of primer pairs pchgms1, pchgms2 and pchcms1, pchcms2, was characterized in peach (*Prunus persica*) by Sosinski *et al.* (2000). The annealing temperature for all primer pairs used in his studies was between 50-60ºC - 30s.

Trying to find the optimum of annealing temperature in our studies, we observed that only pchcms2 primer pair did not generate secondary products at 53ºC, and only when the amplification product was separated in agarose. When the annealing temperature was set up with 5-6 degrees more then Tm, respectively at 64ºC (first 4 probes) and 66ºC (next 4 probes), the PCR product amplified with pchgms2 primer pair did not generated non-specific product in agarose separation.

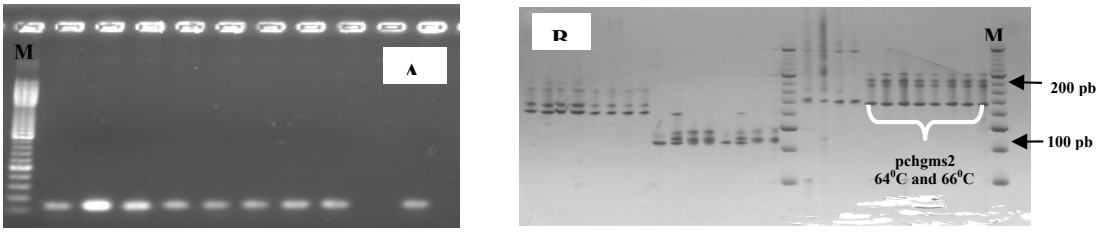

Fig. 2. The PCR product obtained with the primer pair pchgms2 separated in agarose gel (A) and 6%-polyacrylamide gel (B); M-marker of 100 pb DNA ladder (Promega) (A); M-marker of 300 pb DNA ladder (Invitrogen) (B)

In addition the separation of PCR produced in 6%-polyacrylmide gel generated three bands with size range between 170-300 pb (Fig. 2, A and B).

The group of primer pairs UDP was developed by Cipriani *et al.* (1999), to amplify the DNA of eight different species of *Prunus,* including *Prunus cerasus.* The authors recommended 57ºC as an annealing temperature for amplification. Also Zang *et al.* (2008), to

characterize of *Tomentosa cherry* used 110 SSR markers including group of UDP primer pairs.

In our studies using this temperature for amplification, absence of non-specific product was observed at UDP96-001 and UDP96-003 primers pairs only in agarose migration. Increasing the temperature with up to 2-7ºC than Tm, the results were satisfactory also in agarose and 6%-polyacrilamide separation for UDP96-001, UDP96-003 and UDP97-402 primer pairs, but not for UDP97-403. For this primer is necessary to increase the annealing temperature more. In the next figure, we see the absence of non-specific product at separated of PCR amplification by UDP96-003 at 63ºC and 66.2ºC annealing temperature, in agarose and 6%-polyacrylamide gel also. PCR product generated size of fragments between 85-100 pb (Zang *et al.,* 2008). This primer pairs produced polymorphic amplification between varieties (fig.3).

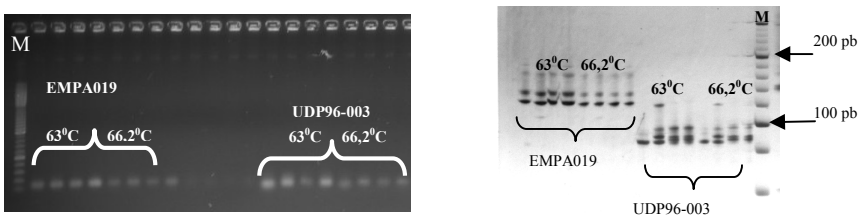

Fig. 3. The PCR product obtained with the primer pair EMPA019 and UDP96-003 separated in agarose gel (left) and 6%-polyacrylamide gel (right); M-marker of 100 pb DNA ladder (Promega) (left); M-marker of 300 pb DNA ladder (Fermentas) (right)

The primer pairs EMPA011 and EMPA019 were used by Clark *et al.* (2003), with success in development and characterization of 14 cultivars of *Prunus avium* and used in PCR amplification in three peach cultivars. For amplification was used touchdown program and 60ºC for 45 sec (-0.5ºC per cycle) as annealing temperature for all primers. In our studies using the same condition, the primer pairs generated secondary products in agarose gel migration. In these conditions, the migration of PCR products in 6%-polyacrylamide gel was not done.

Cantini *et al*. (2001), used SSR primers for fingerprinting of 75 accessions of tetraploid cherry germplasm (sour cherry and ground cherry). He selected primer pairs from different references used for DNA amplification for sweet cherry, sour cherry and peach. We selected from his study the following primer pairs: PMS3, PMS40, PceGa25 and PceGa59. For PCR amplification, Cantini *et al.* (2001), used the annealing temperature for primer pairs of 55ºC for 1 min. Compared with his studies, in our studies when we selected 55ºC as annealing temperature for the primer pairs mentioned above, the results were unsatisfactory. As it is shown in table 3, only primer pairs PceGa25 and PceGa59 generated nonspecific products, as shown by the migration in agarose gel.

A PCR reaction requires primers with similar annealing temperatures, if the two temperatures are very different, amplification will not run properly because the primer with a higher annealing temperature can bind non-specifically if the temperature is optimal for the other primer and this will not remain attached to the first primer specific temperature, which is too high (Sisea and Pamfil, 2009).

Generally accepted rule is to use an initial annealing temperature by 5°C lower than the denaturing temperature. Most of the times this value is not optimal and it is necessary to determine empirically the annealing temperature (Sisea and Pamfil, 2009).

Tab. 2

The results of annealing temperature optimization for 16 pairs of SSR primers

No.	Primer name	Tm(C)* (^{0}C)		Ann. temp. (^{0}C)***	Ann. temp. optimization (^{0}C)	Presence of secondary products		Product size (bp)
		F**	R**			Agarose gel	6% - polyacc. gel	
1.	PS12A02	58.61	60.46	56	55; 56 62; 64 67	+ + -	n.d n.d +	178
2.	pchgms 1	59.87	58.34	50-60	54; 57; 60 63; 66,2	+ -	n.d -	140-190
3.	pchgms 2	58.12	58.23	50-60	55; 57; 60 64; 66	+ -	n.d -	140-190
4.	pchcms1	51.59	56.13	50-60	47; 50 53; 56	+ +	n.d +	166 (130-180)
5.	pchcms2	55.2	55.46	50-60	50; 54 53	+ -	n.d +	180
6.	UDP96-001	53.84	59.13	57	55; 59.3; 62 57 67; 69	+ - -	n.d + -	108-140
7.	UDP96-003	57.43	62.07	57	55; 60 57 63; 66,2	+ - -	n.d + -	135-155
8.	UDP97-402	54.23	60.42	57	55; 57; 60 61,3	+ -	n.d -	130-170
9.	UDP97-403	61.08	60.45	57	55; 57 60; 61	+ -	n.d +	120-150
10.	EMPA011	62.32	60.98	55-60	55 60; 62,7 64; 67	n.a + -	n.d n.d +	240-247
11.	EMPA019	60.73	55.02	55-60	55 60; 61,3; 63,3	- +	+ n.d	101-120
12.	PMS3	55.71	57.13	55	53 55; 57	+ +	+ n.d	152-200
13.	PMS40	56.12	56.2	55-62	53; 55; 57	+	n.d	88-110
14.	PceGA25	57.31	61.43	55	55; 57; 61; 63; 65,1 67	+ + -	+ n.d +	160-200
15.	PceGA50	56.66	61.37	-	58; 60 63; 65	- +	+ n.d	170-175
16.	PceGA59	53.9	55.07	55-62	55; 62; 64 57; 59.3;	- +	+ n.d	180-225

*Tm(C) – melting temperature (Generi Biotech – Czech); ** F-forward and R-reverse

*** Annealing temperature recommended by authors (see table 2); + Presence of secondary products; - absence of secondary products; n.a. – no amplification; n.d.- not done (no migration in 6%-polyacrylamide gel); Bp=base pairs

In our studies, for the optimization of annealing temperature for primer pairs, we tried various stages of annealing temperature from a threshold of 5 degrees lower than the Tm for the primer, as recommended in the literature, up to 6-7 degrees above. Also, a different melting temperature (Tm) for the same set of primers, can generate nonspecific products, and this can be seen in our study. Following the results, from the following sets of primers: pchcms1, UDP96-001, UDP96-003, UDP97-402, EMPA019, PceGA25 and PceGA50, with a Tm difference of about 5 degrees between primers per set, at different annealing temperatures,

after migration in agarose gel, the presence of secondary products can be seen. Instead, for some of these primers, when the annealing temperature was higher than Tm (up to 5^0C), they amplified the target DNA sequence without giving rise to secondary products (the primer set UDP96-001, UDP96-003, UDP97-402) (Tab. 2).

In addition in the table below can be seen the absence of non-specific products after migration of the PCR product in agarose gel and their presence in polyacrylamide gel, for the following set of primers: PS12A02, pchcms2, UDP97-403, EMPA011, PceGA25, PceGA50 and PceGA50.

Usually the length of primers ranges between 16 and 30 nucleotides, and since if the number it is above the annealing (hybridization) is made more difficult - annealing temperature is higher, reaction specificity increases, and product quantity accumulated is reduce (Sisea and Pamfil, 2009).

Sequences of forward or reverse primers used for this study range between 17 and 25 nucleotides, therefore was not necessary to increase the temperature of annealing. On the contrary, when the annealing temperature was increased, observed non-specific PCR products appeared also in agarose and polyacrylamide gel separation, as it can be seen in table 2, for the sets of primers: pchgms1, pchgms2, UDP96-001, UDP96-003 and UDP97-402.

CONCLUSIONS

In the present study, only for 25% from all sets of primers the optimum annealing temperature was found to amplify the sour cherry varieties and indicating transferability from another *Prunus* species.

Similarly, 63ºC and 66.2ºC were found optimum for primer pairs pchgms1 and UDP96-003 respectively; 64ºC and 66ºC for pchgms2; 67ºC and 69ºC for UDP96-001; 61.3ºC for UDP97-402. No more then four fragments were amplified for each varieties/primer pair combination.

Only primer pairs UDP96-003 was the first primers who produced polymorphic amplification between varieties.

These are the partial results in optimizing protocol for annealing temperature in PCR amplification for primer pairs used for fingerprinting of sour cherry from National Collections from Romania.

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