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Molecular-genetic analysis of Azamat pear variety

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Abstract

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In this study, the economic, biological, and morphological characteristics of the newly developed, promising, colorful autumn pear variety, Azamat, were analyzed using molecular genetic methods. The criteria for differentiation, purity, and stability of the variety were also examined. As a result of the genetic analysis, we determined the necessity of adhering to specific varietal characteristics. This variety is distinguished from other pear varieties by a unique combination of alleles at four SSR (Simple Sequence Repeats) loci, confirmed through molecular testing. The purity of the variety was assessed based on the presence of the same allele combination in other tested pear trees. It was also found that the Azamat variety fully matches the standard variety in terms of the allele composition at microsatellite loci. The stability of the variety was confirmed by the presence of identical SSR allele spectra at four loci across different years of reproduction, as determined by molecular genetic testing. If additional alleles appeared in the analyzed sample or if any locus lacked the allele's specific to this variety, it was concluded that the sample did not correspond to the Azamat variety.

Keywords: breeding; hybrid; DNA; PCR; primer; marker

Introduction

The pear is a fruit crop that has been cultivated for many years. It was first introduced to Western Europe (including France, Belgium, Italy, England, and Germany) by the Greeks and Romans. Subsequently, this plant became widely distributed across other European countries, as well as in nearly all regions of North America and Central Asia (Pavlov, 1964). According to available data, the native origin of the genus *Pyrus* is the Caucasus region, located at the boundary between Europe and Asia. From this area, it spread to other geographical regions (Hancock et al., 2008; Naseer et al., 2021). At present, representatives of the genus *Pyrus* are widely distributed across temperate climatic zones and mountainous regions of Asia, Europe, and North America. In addition, pears are cultivated on an industrial scale in more than 50 countries worldwide (Bell 1990; Bell et al., 1996; Erfani-Moghadam, 2012).

According to the literature, representatives of the genus *Pyrus* rank as the third most widely cultivated fruit crops in the world, following grapes and apples (Oliveira et al., 1999; Erfani-Moghadam, 2012). Pear trees can be grown at altitudes of 1,700–2,400 meters above sea level and are distinguished by their better adaptation to favorable climatic con-

ditions compared to other fruit trees. Studies have shown that pear plants can withstand temperatures ranging from −25°C to 45°C during the growing season. It has been reported that relatively warm temperatures and an annual precipitation of about 100 mm are optimal for pear cultivation (Parle et al., 2015; Naseer et al., 2021). Furthermore, pear trees begin to bear fruit 2–3 years after planting, typically reach full bloom within 3–5 months, and are capable of producing yields for up to 75 years (Parle et al., 2015; Naseer et al., 2021).

Today, substantial research has been conducted on pear cultivation in the republic. As a result of long-term studies on pear breeding and variety testing at the Samarkand Scientific Experimental Station, a number of low-growing and high-yielding varieties have been identified (Mirzaxidov, 1985). Nevertheless, some studies indicate that the genetic diversity of pears in Asia remains insufficient. This is explained by the low morphological diversity of representatives of the genus *Pyrus* and the lack of clear distinguishing characteristics between species (Yamamoto et al., 2002).

Morphological traits of pears are crucial for characterizing the genetic diversity of this crop and for determining specific characteristics of pear varieties. However, despite the creation of many varieties, their genetic diversity has not been fully studied or described. Currently, modern molecular markers are being employed to study the genetic diversity of pear species. Among these, markers such as RFLP, RAPD, ISSR, and AFLP are the most widely used (Powell et al., 1996; Iketani et al., 1998; Oliveira et al., 1999; Monte_Corvo et al., 2000; Monte_Corvo et al., 2001; Yamamoto et al., 2001; Teng et al., 2002).

In recent years, SSR markers have been used most frequently in the *Pyrus* genus and are considered the primary source of polymorphisms. Due to the large number and wide distribution of alleles at each locus, they offer reliability in obtaining DNA sequences (Weber et al., 1989; Yamamoto et al., 2002; Erfani-Moghadam, 2012). Additionally, these molecular markers can be used to determine the genetic diversity of pear varieties, study the relationships and origins of representatives of this genus, and differentiate its many varieties (Koller et al., 1993; Hokanson et al., 2011; Baraket et al., 2011; Kong et al., 2011; Uzun et al., 2011; Xie et al., 2011; Erfani-Moghadam, 2012).

In recent years, scientists have identified significant intraspecific polymorphism in a set of biological traits, including several economically valuable traits, as a result of genetic analysis of 93 pear samples. The results indicate a notable change in the morphological characteristics of pear fruits from east to west, as well as an increase in resistance to pathogens and abiotic stress factors (Dagujieva, 2012).

In general, determining the genotypes of fruit plants is a highly relevant issue in modern horticulture, and is considered a key factor in protecting intellectual property, as well as in maintaining varietal purity and selection achievements of individual fruit seedlings (Powell et al., 1996; Iketani et al., 1998; Oliveira et al., 1999; Monte_Corvo et al., 2001).

To date, there has been insufficient scientific research on the genetic diversity of pear varieties common in our republic. Therefore, in our study, we aimed to investigate the genetic diversity of newly created pear varieties and develop their identification using molecular genetic methods.

Materials and Methods

For the research, the newly created Azamat and Farizi varieties from the Samarkand Scientific-Experimental Station of the Scientific-Research Institute of Horticulture, Viticulture and Winemaking named after Academician M. Mirzaev, as well as 5 varieties and hybrids from the collection gardens, such as Zuhra, Durdona and Lastochka, were used. "Invitrogen PureLink™ Genomic DNA Mini Kit" (Thermo Fisher USA) reagents were used to isolate DNA from the leaves of samples taken from these varieties. The DNA isolation procedure was carried out based on the methodological instructions of the "Invitrogen PureLink™ Genomic DNA Mini Kit".

For DNA extraction, samples of young leaves were collected, placed in a mortar, and thoroughly homogenized using liquid nitrogen. A 200 mg portion of the prepared homogenized mass was transferred to a 1.5 ml Eppendorf tube, and 180 µl of PureLink™ Genomic Digestion Buffer was added and vortexed. Then, 20 µl of Proteinase K was added to the mixture to digest proteins, and the sample was incubated for 2 hours at 55°C in a thermostat. The mixture was shaken every 10 minutes during incubation.

The mixtures were removed from the thermostat, and 200 µl of PureLink™ Genomic Lysis/Binding Buffer was added. The samples were then centrifuged at 10.000 rpm for 5 minutes. The supernatant was carefully transferred into new tubes. To each tube, 200 µl of 96–100% ethanol was added, and the mixture was vortexed. The prepared lysate was placed in a column, and centrifuged again at 10,000 × g for 1 minute. Then, 500 µl of PureLink™ Genomic Wash Buffer (I) was added to wash the extracted DNA, followed by centrifugation at 10.000 × g for 1 minute. The liquid at the bottom of the column was discarded, and the column was transferred to a new tube. Next, 500 µl of PureLink™ Genomic Wash Buffer (II) was added, and the mixture was centrifuged at 10,000 × g for 3 minutes. The column was then centrifuged at 15.000 × g for 2 minutes to remove any residual wash buffer. Finally, the column was transferred to a new 1.5 ml Eppendorf tube.

Next, 100 µl of PureLink™ Genomic Elution Buffer was added to the membrane of the column and incubated for 2 minutes at room temperature. The column was then centrifuged at 10.000 rpm for 2 minutes. The isolated DNA was collected in the eluate, and the pellet was discarded. The isolated DNA was stored at -20°C.

DNA quality was assessed by electrophoresis on a 0.9% agarose gel. The concentration of DNA isolated from pear varieties was measured using a Qubit 2.0 Fluorometer (Invitrogen, Thermo Fisher, USA). Qubit® dsDNA HS Assay Kits, with a high-precision DNA concentration range of 10 pg/µl to 100 ng/µl, were used for the measurement. The concentration determination procedure was carried out according to the kit instructions.

Results and Discussion

Our analyses highlighted the advantages of the SSR-marking method and its effectiveness in identifying agricultural crop varieties. The high diversity of SSR locus alleles in the pear genome, coupled with the elevated level of polymorphism in representatives of the Pyrus genus, makes it possible to use a broad set of markers for genotype identification through SSR analysis.

The results of the studies showed that PCR products were obtained for all samples at the SSR marker loci. Significant differences were observed between the varieties and genotypes analyzed.

Four highly polymorphic SSR markers – CH01d09, SdSSR, CH02c02b, and CH04h02 were selected to create a genetic passport for the Azamat variety. The characteristics of these SSR markers, including chromosomal localization, allele size range, and number of alleles in the pear genome, are presented in Table 1.

Within the framework of this study, the nucleotide sequences of SSR markers were identified in FASTA format, and their applicability in molecular genetic analyses was evaluated. In particular, the sequences obtained for the markers CH01d09, SdSSR, CH02c02b, and CH04h02 enabled precise identification of specific genomic regions and their comparison. This facilitated the reliable detection of molecular differences among various genotypes during the study. The use of FASTA-formatted data allowed for the standardization of markers and their comparison with international databases. As a result, these SSR markers were found to provide a high level of reproducibility and accuracy. This indicates their effectiveness as tools for assessing genetic diversity, identifying genotypes, and conducting phylogenetic analyses.

PCR parameters

The SSR (Simple Sequence Repeat) markers used in this study demonstrated high efficiency in the molecular-level assessment of pear (*Pyrus*) genotypes. The primers applied for the analyzed markers – CH01d09, SdSSR, CH02c02b, and CH04h02 – enabled the amplification of specific genomic regions and ensured reliable detection of genetic variation. The labeling of all forward primers with the ROX fluorescent dye allowed for high-precision detection of amplicons through capillary electrophoresis, thereby increasing the reliability of the obtained results (Table 2).

Table 2. List of primers used in the study

CH01d09.F	5’-ROX-GCCATCTGAACAGAATGTGC-3’
CH01d09.R	5’-CCCTTCATTCACATTTCCAG-3’
SdSSR.F	5’-ROX-GAATTCTCGTCCCTTCATCTC-3’
SdSSR.R	5’-GTTCCTTAGCCTCCCATTCTG-3’
CH02c02b.F	5’-ROX-TGCATGCATGGAAACGAC-3’
CH02c02b.R	5’-TGGAAAAAGTCACACTGCTCC-3’
CH04h02.F	5’-ROX-GGAAGCTGCATGATGAGACC-3’
CH04h02.R	5’-CTCAAGGATTTCATGCCCAC-3’

Source: Authors’ own elaboration

The findings indicate that the selected SSR markers possess high informativeness in detecting genomic polymorphism and serve as effective tools for evaluating genetic diversity among pear varieties. This highlights their significant potential for application in breeding programs, including the conservation of valuable agronomic traits and the development of new cultivars.

PCR was performed using the primers listed in Table 3 and the Platinum™ Hot Start PCR Master Mix (2X) reagent

Table 1. Description of selected SSR markers

№	Marker name	Chromosome	Allele size range in the pear genome, *nucleotide pairs (n.p.)*	Number of detectable alleles in the pear genome
1	CH01d09	12	128–168	18
2	SdSSR	7	140–206	14
3	CH02c02b	4	109–133	8
4	CH04h02	11	160–202	13

Source: Authors’ own elaboration

kit, which contains a ready-to-use mixture of DNA polymerase, salts, magnesium, and dNTPs.

The PCR amplification was performed using the following program: an initial denaturation at 95°C for 11 minutes, followed by 32 cycles of denaturation at 94°C for 20 seconds, annealing and elongation at 56°C, and a final elongation at 56°C for 60 minutes.

Polymerase chain reaction (PCR) was carried out using a MiniAmp™ Plus Thermal Cycler (Applied Biosystems, USA). Fragment analysis was performed on an ABI PRISM® 3500 Genetic Analyzer, and the obtained data were analyzed using GeneMapper® ID-X software (version 1.4).

The GeneScan™ 600 LIZ® Size Standard was used to determine the size of DNA fragments. This standard is specifically designed for accurate sizing of DNA fragments within the 20–600 nucleotide range. It consists of 36 single-stranded fluorescently labeled fragments with defined lengths of 20, 40, 60, 80, 100, 114, 120, 140, 160, 180, 200, 214, 220, 240, 250, 260, 280, 300, 314, 320, 340, 360, 380, 400, 414, 420, 440, 460, 480, 500, 514, 520, 540, 560, 580, and 600 nucleotides. The electropherogram profiles and fragment distribution patterns obtained using the GeneScan™ 600 LIZ® Size Standard are presented in Fig. 1, Fig. 2, and Fig. 3, respectively.

Fragment analysis of the Azamat pear variety using four SSR markers identified distinct allelic profiles, as presented in Figures 4, 5, 6, and 7. The application of these markers enabled the detection of polymorphic loci and provided insight into the genetic structure of the studied genotype. Each SSR marker revealed specific allele sizes, reflecting variations within the genomic regions analyzed and confirming their suitability for molecular characterization.



Fig. 1. The format of shown primers positions
Source: Authors' own elaboration

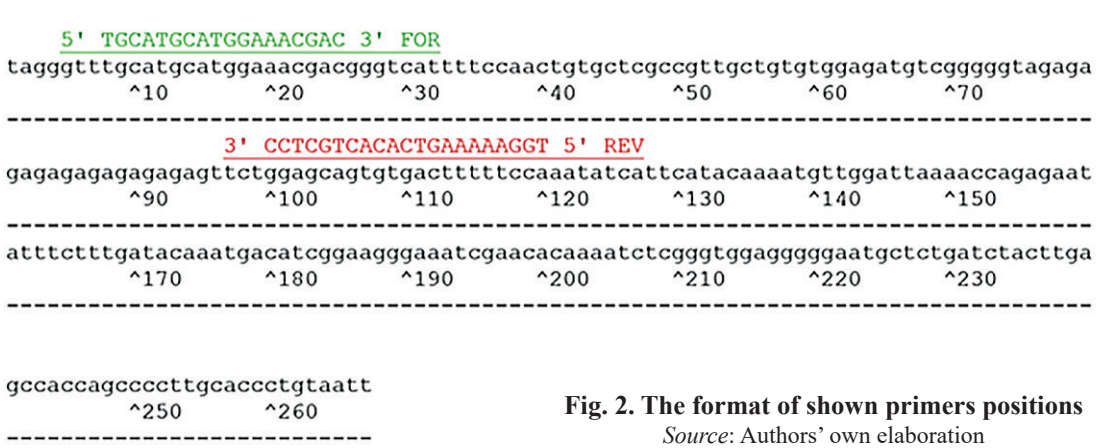


Fig. 2. The format of shown primers positions
Source: Authors' own elaboration

```
PCRStart  PCREnd
                                        5' GCCATCTGAACAGAATGTGC 3' FOR
aattccaactaggtgaaaggaggtggtttgacaatgccatctgaacagaatgtgcatctttttcttggcaaataagagga
         ^10       ^20       ^30       ^40       ^50       ^60       ^70
-------------------------------------------------------------------------------

atagtgaaatatggagagagagagagagagagagagagagagagagagagagagatttgcttggcaggggangaaaaat
         ^90       ^100      ^110      ^120      ^130      ^140      ^150
-------------------------------------------------------------------------------------
3'GACCTTTACACTTACTTCCC 5' REV
anctggaaatgtgaatgaagggagtggggttncgtntaagtgaggggaatgatggggtttttttgcttctgttttggtc
         ^170      ^180      ^190      ^200      ^210      ^220      ^230
-------------------------------------------------------------------------------


caacttcaaanaaaaaagcttgcagttaaagtattttcattacaagcagttaggatgggaccaatt
         ^250      ^260      ^270      ^280      ^290      ^300
-------------------------------------------------------------
```

Fig. 3. The format of shown primers positions

Source: Authors' own elaboration

The observed allelic patterns demonstrate the effectiveness of SSR markers in distinguishing genotypes at the DNA level, even in cases where morphological traits may appear similar. This highlights their importance for precise identification and classification of pear varieties. Furthermore, the consistency and clarity of the fragment profiles indicate a high level of reliability and reproducibility of the applied methodology.

Based on SSR analysis, a molecular genetic passport was created for the Azamat variety, representing it as a set of alleles at four SSR loci. The allele composition of the Azamat variety at these loci is presented in Table 4. As shown in Table 4, the genotyped Azamat variety possesses a unique set of alleles, identified through a combination of four SSR markers.

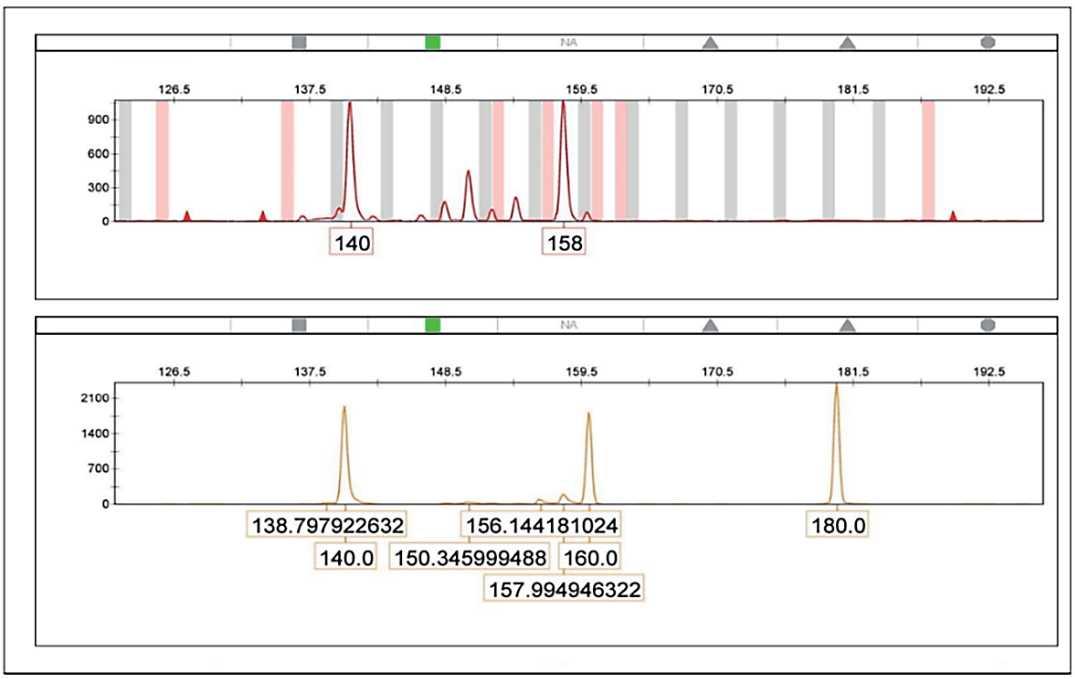


Fig. 4. A – Allelic profile of the Azamat variety based on the CH01d09 SSR marker (140, 158). B – GeneScan™ 600 LIZ® size standard

Source: Authors' own elaboration

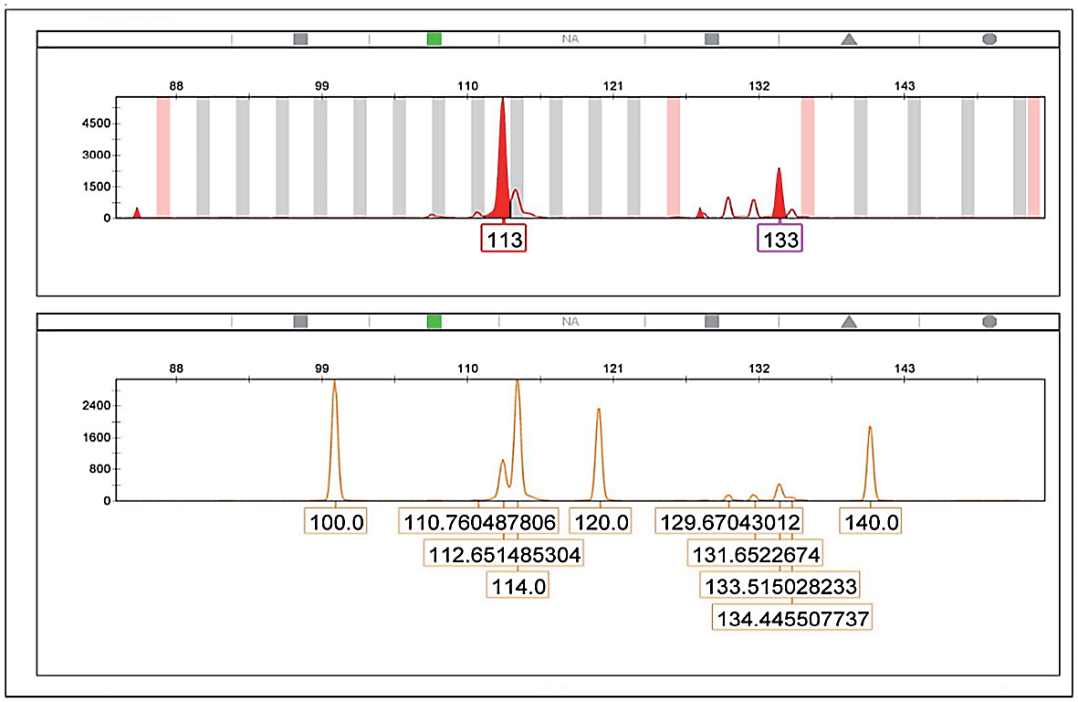


Fig. 5. A – Allelic profile of the Azamat variety based on the SdSSR SSR marker (160).B – GeneScan™ 600 LIZ® size standard
Source: Authors' own elaboration

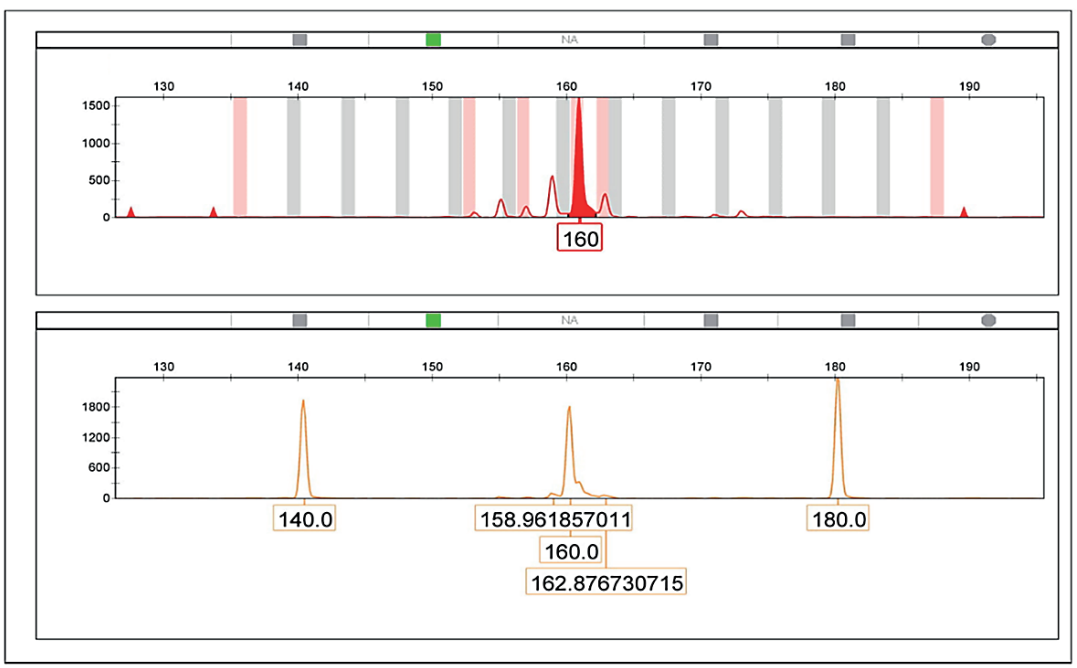


Fig. 6. A – Allelic profile of the Azamat variety based on the CH02c02b SSR marker (113, 133). B – GeneScan™ 600 LIZ® size standard
Source: Authors' own elaboration

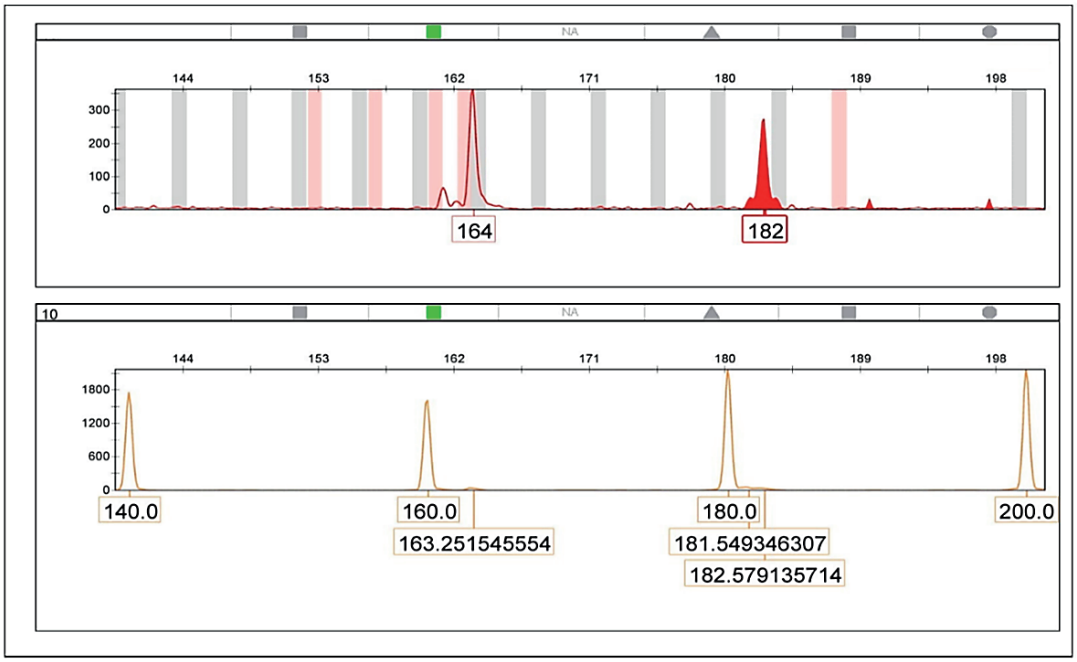


Fig. 7. A – Allelic profile of the Azamat variety based on the CH04h02 SSR marker (164,182). B – GeneScan™ 600 LIZ® size standard

Source: Authors' own elaboration

Table 3. Components of the PCR reaction mixture (25 µL total volume)

Components	Quantity, µL
ddH2O	7,8 µL
Platinum™ Hot Start PCR 2X Master Mix (Master mix mixture)	12,5 µL
10 µM forward primer	1,0 µL
10 µM reverse primer	1,0 µL
DNA	2,7 µL
Total	25,0 µL

Source: Authors' own elaboration

Table 4. Molecular genetic passport of the Azamat variety based on SSR analysis

Variety name	Allelic sizes of SSR markers			
	CH01d09	SdSSR	CH02c02b	CH04h02
Azamat	140, 158	160	113, 133	164,182

Source: Authors' own elaboration

Table 5. Comparative analysis of the allelic composition of the Azamat variety with other pear varieties

№	Variety name	Origin	CH01d09	SdSSR	CH02c02b	CH04h02
1	Azamat	Uzbekistan	140, 158	160	113, 133	164,182
2	Allegro	Russia		140, 164	133	188
3	Belorusskaya pozdnyaya	Belarus		168, 176	117, 129	184, 190
4	Bere loshiskaya	Belarus		166, 172	119, 125	184
5	Bere Lyuka	France	131, 133, 135	164	125, 129	184
6	Bolshaya letnyaya	Ukraine		162, 164	119, 125	184
7	Velesa	Russia		158, 164	133	162, 202

Table 5. Continued

№	Variety name	Origin	CH01d09	SdSSR	CH02c02b	CH04h02
8	Vernaya	Russia		158, 164	133	176, 184
9	Vesnyanka	Russia		154, 206	125, 133	184
10	Vidnaya	Russia		154, 174	119	184
11	Viktoriya	Ukraine		154, 184	119, 133	184
12	Vrodliva	Ukraine		164, 184	119, 125	164
13	Vistavochnaya	Ukraine		154, 206	133	184
14	Gibrid 25	Russia		162, 184	117, 119	184
15	Desertnaya rossoshanskaya	Russia		170	125, 133	164, 184
16	Duxmyanaya	Belarus		164	117, 133	172, 184
17	Dyushes letniy	Belarus		170, 184	129, 133	184, 194
18	Eseninskaya	Russia		158, 174	133	184, 194
19	Zabava	Belarus		164, 184	119, 133	164, 184
20	Zolotovorotskaya	Ukraine		174	109, 117	184
21	Kolxoznitsa	Belarus		154, 174	119, 125	184
22	Konferensiya	England	155, 161	158, 172	133	184
23	Krasavitsa Chernenko	Russia		154, 174	133	184
24	Kudesnitsa	Belarus		154, 176	125, 133	182, 202
25	Lagodnaya	Belarus		154, 176	117, 133	184
26	Lira	Russia		164	119, 125	164, 184
27	Maslyanaya Ro	Ukraine		164, 206	133	184
28	Mlievskaya rannyaya	Ukraine		154, 176	133	184
29	Mramornaya	Russia		166, 172	129, 133	164, 184
30	Muratovskaya	Russia		164	117	164, 176
31	Naryadnaya Yefimova	Russia		154, 172	117	184, 202
32	Noyabrskaya	Moldova		162, 206	119, 133	184
33	Obrazes № 12	Poland		162, 184	119, 125	162
34	Pamyati Yakovleva	Russia		154, 178	133	184
35	Pin-go-li	China		140, 154	111	166, 186
36	Svetlyanka	Russia		140, 154	119	184
37	Silva	Ukraine		154, 206	119, 133	184
38	Sladkaya iz Mlieva	Ukraine		154, 168	133	172, 184
39	Sokroviщe	Ukraine		154	133	184
40	Talgarskaya krasavitsa	Kazakhstan		176	111, 133	176, 184
41	Xoni Dyu	USA		168, 184	111, 117	184, 188
42	Chijovskaya	Russia		140, 164	119, 121	188, 202
43	Yurate	Lithuania		162, 170	129, 133	164, 184
44	Yasachka	Belarus		168, 172	117, 133	182, 190
45	Abbe Fetel	France	155, 157	No data	No data	No data
46	Bartlett	Europe	153, 161	No data	No data	No data
47	Doyenne du Comice		137, 155	No data	No data	No data
48	Hosui		157, 161	No data	No data	No data
49	Passe Crassane		137, 155	No data	No data	No data
50	Pendula		143, 153	No data	No data	No data

Source: Authors' own elaboration

According to the results of the comparative analysis, the allelic composition (combination) of the Azamat variety was not found in 50 pear varieties from different countries. This confirms the effectiveness of the selected SSR marker set and highlights the distinctiveness and uniqueness of the Azamat variety (Table 5).

Conclusion

Overall, the set of four SSR markers selected in this study can be effectively used to identify the Azamat variety. The genotype of this variety is characterized by a unique combination of alleles detected using these four SSR markers.

The proposed DNA identification method for the Azamat variety meets the criteria for differentiation, purity, and stability in molecular genetic testing. We suggest that genetic analysis of this variety should adhere to the following varietal characteristics:

– **Differentiation**: The Azamat variety is distinct from other pear varieties due to its unique combination of alleles at four SSR loci, as confirmed by molecular genetic testing.

– **Purity**: All fruit trees of the Azamat variety exhibit the same combination of alleles at the studied loci. Since pear varieties reproduce vegetatively, each individual plant or its parts must fully match the Azamat standard variety in terms of allele composition at microsatellite sequence loci. If a pear tree undergoing molecular genetic testing contains alleles that deviate from the standard variety, the sample should be considered non-varietal. Therefore, each individual pear tree and sapling must undergo molecular genetic testing.

– **Stability**: The Azamat pear variety must maintain the same SSR allele profiles at four loci across different years of reproduction, as verified by molecular genetic testing. If additional alleles appear in the analyzed sample or if any alleles specific to this variety are absent at any locus, the sample is deemed incompatible with the Azamat variety.

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