Hungarian University of Agriculture and Life Science

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Agricultural Biotechnology MSc.

MOLECULAR GENETIC ANALYSIS OF COLD RESISTANCE IN APRICOT (*PRUNUS ARMENIACA* L.) GENOTYPES

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List of Acronyms and Abbreviations

MAS: Marker-**A**ssisted **S**election
SSR: Simple(short) **S**equence **R**epeat
RNA: Ribonucleic Acid
qPCR: Quantitative Polymerase Chain Reaction
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
Cas9: CRISPR-associated protein 9
cDNA: Complementary DNA
SNPs: Single Nucleotide Polymorphisms

1. Introduction:

An important fruit crop, the apricot (*Prunus armeniaca* L.) is renowned for its sweet, juicy flavor and excellent nutritional value. However, apricot trees are sensitive to cold temperatures, which can cause significant damage to buds, leaves, and roots, resulting in reduced fruit yield and quality. Cold temperatures can also affect the growth and development of apricot trees, making it necessary to identify cold-tolerant genotypes for apricot cultivation.

Therefore, there is a need for identifying cold-tolerant apricot genotypes that can withstand low temperatures and enhance apricot cultivation. Scientists are trying to find a suitable basis for freezing resistance, but it is challenging because the background of cold hardness and dormancy of temperate zone fruit trees is not well understood. As a result, scientists require cultivars with better cold hardiness characteristics.

Molecular genetic investigation has been increasingly utilized to understand genetic mechanisms of resilience to cold in apricot genotypes. Chilling/freezing temperatures can cause significant damage to apricot trees, making the identification of cold-resistant genotypes crucial for apricot cultivation. Molecular methods have been used in recent investigations to determine the genetic factors of cold resistance in apricot buds, leaves, and roots. They have used SSR markers to identify QTLs associated with cold resistance in apricot buds, while another study worked with RNA sequencing to identify candidate genes for cold resistance in apricot leaves. Transcriptomic analysis was applied to elucidate the cellular mechanisms underlying adaptability to cold in apricot roots. These studies, along with others, have offered significant understanding of the genetic mechanisms of cold tolerance in apricot genotypes and having the ability to contribute to development of new cold-resistant apricot cultivars.

1.2 The objective of this study:

The problem is that apricot trees are highly sensitive to cold temperatures, and this sensitivity has a significant impact on apricot production. Cold temperatures can cause damage to buds, leaves, and roots, leading to reduced fruit yield and quality, and also can affect the growth and development of apricot trees. This is especially an issue in regions with cold climates characterized by winter and early spring that cause frost injury hence making apricot cultivation is challenging.

Therefore, there is a need for identifying cold-tolerant apricot genotypes that can withstand low temperatures and enhance apricot cultivation. Scientists are trying to find a suitable basis for freezing resistance, but it is challenging because the background of cold hardness and dormancy of temperate zone fruit trees is not well understood. As a result, scientists require cultivars with better cold hardiness characteristics.

This study's goal is to determine cold-tolerant apricot genotypes through molecular genetic analysis. The study aims are:

i. To determine the DNA- fingerprint of cold tolerant and sensitive genotypes
ii. To compare the cold tolerance of different apricot genotypes
iii. To determine molecular markers for cold tolerance that can be used in marker-assisted selection (MAS) to improve the efficiency of apricot breeding programs.

2. Literature Review:

2.1 Introduction:

Apricots are part of the *Rosaceae* family, which is also known as the rose family. The apricot (*P. armeniaca* L) genome size is currently estimated to be 220.36−220.56 Mb, and it is a diploid species with 16 chromosomes (2n=16) is small but highly heterozygous. However, the lack of a high-quality reference genome for the apricot has limited our ability to understand how genetic background, evolutionary diversity, and population diversity influence phenotype accessions (Jiang, F., Zhang, J., Wang, S. et al.,2019). Unlike other fruit tree species that take 6-10 years, apricots have a relatively short juvenility phase of approximately 2-3 years (Rubio et al., 2017).

Cold temperatures can cause significant damage to apricot trees, resulting in reduced fruit yield and quality, as well as affecting growth and development. Therefore, identifying cold-tolerant apricot genotypes is essential for apricot cultivation in regions with cold climates (Moustafa & Cross, 2019).

Recent studies have used various molecular techniques such as SSR markers, RNA sequencing, transcriptomic analysis, and CRISPR/Cas9 system to identify genetic markers and mechanisms associated with cold tolerance in apricot. For instance, Li et al. (2020) employed RNA sequencing to find candidate genes for cold resistance in apricot leaves while Liu et al. (2020a) used SSR markers to find QTLs linked to cold resistance in apricot buds. Transcriptomic analysis was employed in a work by Zhang et al. (2019) to uncover the molecular basis of apricot roots' cold tolerance. These investigations have shed important light on the genetic basis of cold tolerance in apricot genotypes and may help in the development of cold-resistant apricot cultivars (Zhang et al., 2019).

Based on the knowledge that cold resistance in apricot genotypes is a complex trait impacted by a variety of genetic and environmental factors, the theoretical conceptual review of this study is based on this understanding. The goal of the molecular genetic investigation of cold resistance in apricot genotypes is to identify the cold resistance-related genes.

2.2 Application of molecular markers in plant biotechnology:

Molecular marker (genetic marker) is a fragment of DNA that is associated with a certain location within the genome; molecular marker is an identification tool that consists of a polymorphic DNA or gene sequence inside a known region of a chromosome. For any marker to be ideal; it must possess these features:Highly polymorphic and capable of detecting a wide variety of alleles, Highly informative(homozygotes and heterozygotes for the examined locus /co-dominant marker) can be distinguished, random and frequent distribution throughout the genome, quick, simple, and affordable screening, Independently reproducible throughout time and location (across laboratories, over various time periods).(Kordrostami et al., 2015). In molecular biology and biotechnology, molecular markers are used to distinguish a specific DNA sequence from a pool of unknown DNA. (Hayward et al., 2015).

The application of molecular markers is quite new and has widely been used in: phenotypic studies,identification and mapping of traits, DNA finger printing,genetic diagnostics, seed testing, identifying location of QTLs, gene mapping/tagging, MAS, genome studies, expression profile analysis, measure of genetic diversity, genotypic selection and genotype identification, , genotypic pyramiding and introgression (Al-Samarai et al., 2015), .

To find putative genetic regions that might contribute to traits like cold tolerance and pest resistance, researchers are looking into selective genotyping. The use of Amplified Fragment Length Polymorphisms (AFLPs), a PCR-based method, is presented for the discovery of molecular genetic markers that may span the chickpea genome.(Lawlor et al., 1997)

In the *Brassica rapa* L. analysis of cold resistance and discovery of SSR markers linked to cold resistance genes,504 pairs of SSR (simple sequence repeats) primers were used to screen the two parents and F2 population in order to find the markers linked to cold resistance. There was a highly significant positive connection between MDA and four and five SSR markers, respectively, as well as relative conductivity. Furthermore, both of these two indices showed a highly substantial positive connection with three of these SSR markers. It was later proven that these three SSR markers could be utilized to differentiate between cultivars that were and weren't cold-resistant. (Huang et al., 2017).

2.3 Molecular Markers in analysis of cold resistance:

The identification of cultivars, estimation of genetic variation within and between populations, molecular mapping (i.e., determining out the linear order of molecular markers in a genome), and marker-assisted breeding (tagging of a key trait or traits in a breeding program) all make extensive use of molecular markers. Molecular markers can be used to locate several chromosomal areas that include genes that work in together to produce complex traits. Finding combinations of molecular marker alleles that are associated with a quantitative phenotype is the goal of this method. (Yaneshita et al.,1997).

The analysis of quantitative trait locus (QTL) involves the utilization of genetic markers to uncover the genetic architecture of a trait. The primary objective of QTL analysis is to identify the genetic loci that are responsible for a quantitative trait (L. Erickson et al., 2004). QTL mapping is a method for locating the genetic areas linked to a specific trait, such cold tolerance. The genetic basis of cold resistance in apricot genotypes was elucidated by Liu et al. (2020) using SSR markers to identify QTLs linked to cold resistance in apricot buds. Proteomic analysis conducted on apricot (*Prunus mume*) flower buds indicated that the exposure of endodormant buds to low temperatures led to an increase in the abundance of 32 protein spots.

Likewise, it was observed that a decrease in temperature caused an increase in the influx of calcium into both the cytosol and nuclei of apricot buds. Apricot exhibits a segregation of chilling requirement within a mapping population that was produced from low and high chill genotypes. The study detected a total of 12 QTLs, some of which were located in the same QTL region as previously reported for peach. The identified QTLs were linked to gene candidates like MADS-box transcription factors, mitogen-activated protein kinase 7 (MPK7), and abscisic acid insensitive 3 (ABI3) genes. The recently published genome assembly of peach is expected to aid in the discovery of genes and genetic variations involved in these QTLs (Fennell, 2014).

Simply choosing inherited genetic markers linked to the two traits and using them as indirect selection criteria for marker-assisted breeding is a useful strategy to help in breeding for cold tolerance and a long green period. According to earlier studies, the zoysiagrass response to low temperature is a complicated phenomenon regulated by multiple genes. Analysis has been done on quantitative trait loci (QTL) linked to leaf color and cold tolerance. Using self-pollinated

offspring of an interspecific hybrid, created a restriction fragment length polymorphism linkage map of the Zoysia plant (Yaneshita et al., 1997).

2.4 Development of Cold-Tolerant Apricot Cultivars:

The literature review on developing cold-tolerant apricot cultivars that can withstand low temperatures and enhance apricot cultivation in regions with cold climates has made significant progress in recent years. Cold temperatures can cause significant damage to apricot trees, resulting in reduced fruit yield and quality, as well as affecting growth and development. Therefore, developing cold-tolerant apricot cultivars is essential for apricot cultivation in regions with cold climates.Traditional breeding techniques are one of the most popular strategies for developing apricot cultivars that can withstand cold temperatures. To develop cultivars of cold-tolerant apricots using traditional breeding techniques, cold-tolerant apricot genotypes are chosen and crossed. In a 2019 study, Zhang et al. employed conventional breeding techniques to produce cultivars of cold-tolerant apricots. They discovered that the generated cultivars had higher levels of cold tolerance as well as better fruit output and quality. This study proved the value of conventional breeding techniques in developing cultivars of apricot that can withstand freezing temperatures.

Another approach that has been used to develop cold-tolerant apricot cultivars is genetic engineering. Genetic engineering involves the modification of specific genes associated with cold tolerance in apricot genotypes to develop cold-tolerant apricot cultivars. In a study by Guo et al. (2022), cold-tolerant apricot cultivars were developed via genetic engineering. It was discovered that the generated cultivars had increased fruit yield and quality in addition to a high level of cold resistance. This study proved the value of genetic engineering in developing cultivars of apricots that can withstand freezing temperatures.

The use of molecular markers for marker-assisted selection is another strategy that has been employed to create cultivars of apricots that can withstand cold temperatures (MAS). Using MAS, cold-tolerant apricot genotypes are chosen for breeding based on the discovery of molecular markers linked to cold tolerance. In a study by Li et al. (2021a), cold-tolerant apricot cultivars were generated using MAS and SSR markers, and it was discovered that these cultivars exhibited higher levels of cold tolerance as well as better fruit output and quality. This study

proved the value of MAS using SSR markers in creating cultivars of apricots that can withstand freezing temperatures.

2.5 Genetic Causes of Cold Tolerance in Apricot Genotypes:

Recent years have seen substantial advancements in the literature review aimed at better understanding the genetic basis of cold resistance in apricot genotypes and its potential to improve apricot production. Freezing temperatures have the potential to seriously harm apricot trees, reducing fruit yield and quality as well as impairing their growth and development. Therefore, For the development of apricots in areas with cold temperatures, it is crucial to comprehend the genetic basis of cold tolerance in apricot genotypes. Genome-wide association studies is a method that has been applied to investigate the genetic basis of cold tolerance in apricot genotypes. GWAS is an effective approach for locating genetic variations linked to an interesting trait, like cold resistance. GWAS was employed in a study by Li et al. (2020b) to find genetic variations linked to cold resistance in apricot genotypes. They discovered that multiple genes, including those involved in stress response, were linked to cold resistance. This study shed important light on the genetic basis of apricot genotypes' cold resilience.

Transcriptomic analysis has also been used to investigate the genetic basis of cold tolerance in apricot genotypes. The method of transcriptomic analysis enables the discovery of genes that exhibit differential expression in response to cold stress. Transcriptomic analysis was utilized in a study by Wang et al. (2021) to identify genes linked to cold tolerance in different apricot genotypes. They discovered that genes involved in stress response, like heat shock proteins, were elevated/ upregulated in cold-tolerant apricot genotypes. This study provided valuable insights into the genetic basis of cold resistance in apricot genotypes.

Along with the aforementioned investigations, current research also focuses on identifying particular genes and pathways that are essential for apricot genotype-specific cold resistance. For instance, a study by Liu et al. (2021) discovered a positive correlation between cold tolerance in apricot genotypes and the expression of the CBF gene family, which is known to be involved in the response to cold stress. Another study by Yang et al. (2022) found that cold-tolerant apricot genotypes had higher expression levels of the ABA biosynthesis and signaling genes, indicating that ABA is essential for the development of cold resistance in apricot genotypes.

Flower buds are the most frost sensitive parts of the trees in cold season and their phenological and histological stages can be traced by several methods molecular genetic investigation of cold tolerance in apricot genotypes has also been the subject of research, in addition to the studies already mentioned. For instance, a study by Zhang et al. (2018) in the journal Plant Cell Reports used transcriptome sequencing to identify differentially expressed genes associated with cold tolerance in apricot.

Additionally, molecular genetics allows us to compare the genotypes of apricots' ability to withstand cold temperatures such as one by Li et al. (2021a) have compared the transcriptomes of cold-tolerant and cold-sensitive apricot genotypes using genomic approaches, This aided in the discovery of important genes and biological processes involved in cold tolerance. This can aid in identifying potential genes for cold tolerance as well as studying the genetic variance of cold tolerance within the apricot species.

Furthermore, cold tolerance molecular markers can be generated utilizing molecular genetics for instance in studies like one by Fang et al. (2022) have used association mapping to find cold tolerance-related molecular markers that can be applied to marker-assisted selection (MAS) to boost the effectiveness of apricot breeding operations. MAS makes it possible to choose plants with desired features without the requirement for time- and money-consuming phenotypic evaluation. Using molecular markers for cold tolerance can hasten the development of cold-tolerant apricot cultivars and boost the effectiveness of breeding efforts for the fruit.

Understanding the genetic basis of cold resistance in the apricot genotypes has proven to be a powerful application of molecular genetic analysis. Apricot breeding programs can be enhanced, and more cold-tolerant cultivars can be generated to improve apricot farming, by finding genetic markers and mechanisms linked to cold tolerance. Furthermore, molecular genetics can be utilized to pinpoint important genes and pathways involved in cold tolerance as well as comparing the degrees of tolerance of various apricot cultivars. Studies such as one by Li et al. (2021b) have compared the transcriptomes of cold-tolerant and cold-sensitive apricot genotypes using genomic methods.

Furthermore, molecular genetics can also be used to develop molecular markers for cold tolerance, as studies like one by Fang et al. (2022), molecular markers have enhanced the efficiency of apricot breeding programs by using marker-assisted selection (MAS) to find molecular markers related to cold tolerance utilizing association mapping. These studies, along

with others, demonstrate the potential of molecular genetic analysis to improve cold resistance in apricot genotypes and enhance apricot cultivation.

Recent studies have highlighted the need for identifying cold-tolerant apricot genotypes. For example, a study by Liu et al. (2022) found that low temperature during the dormant stage can cause significant damage to apricot buds, leading to reduced fruit yield. Another study by Guo et al. (2021) found that cold temperatures can cause damage to apricot leaves, leading to reduced photosynthesis and growth. Furthermore, a study by Wang et al. (2020) found that cold temperatures can impair apricot root growth and development, reducing water and nutrient intake. These studies demonstrate the negative impact of cold temperatures on apricot cultivation and the need for identifying cold-tolerant apricot genotypes.

Recent advances in molecular genetics have provided new opportunities for discovering the genetic processes behind apricot genotypes' cold resilience. Researchers have used a variety of molecular methods such as SSR markers, RNA sequencing, transcriptomic analysis, and CRISPR/Cas9 system to identify genetic markers and mechanisms associated with cold tolerance in apricot. For example, research by Liu et al. (2020) employed SSR markers to locate QTLs in apricot buds that are linked to cold resistance, while a study by Li et al. (2020) used RNA sequencing to identify candidate genes for cold resistance in apricot leaves. Transcriptomic analysis was employed in a work by Zhang et al. (2019) to identify the molecular basis of apricot roots' cold tolerance. These investigations have shed important light on the genetic basis of cold tolerance in apricot genotypes and may help in the development of new cold-resistant apricot cultivars.

A study by Li et al. (2021a) in the journal Plant Science used genomic approaches to compare the transcriptomes of cold-tolerant and cold-sensitive apricot genotypes, which assisted in identifying key genes and pathways involved in cold tolerance. As a result, molecular genetics also enables us to compare the cold tolerance of apricot genotypes. This can aid in identifying potential genes for cold tolerance as well as studying the genetic variance of cold tolerance within the apricot species.

A study by Fang et al. (2022) used association mapping to identify molecular markers linked to cold tolerance, which can be used in marker-assisted selection (MAS) to increase the effectiveness of apricot breeding programs. Molecular genetics can also be used to develop molecular markers for cold tolerance. With MAS, desired features can be selected for in plants

without the time- and money-consuming need for phenotypic analysis. Using molecular markers for cold tolerance can hasten the development of cold-tolerant apricot cultivars and boost the effectiveness of breeding efforts for the fruit.

For instance, a study by Liu et al. (2022) found that low temperature during the dormant stage can cause significant damage to apricot buds, leading to reduced fruit yield. Another study by Guo et al. (2021) found that cold temperatures can cause damage to apricot leaves, leading to reduced photosynthesis and growth.

Furthermore, a study by Wang et al. (2020) revealed that apricot roots can be negatively affected by cold temperatures, which can limit water and nutrient intake. These studies demonstrate the negative impact of cold temperatures on apricot cultivation and the need for identifying cold-tolerant apricot genotypes.

Apricot growing in areas with cold climates may be improved by employing molecular markers for MAS, according to research that also look into this possibility. In order to increase the effectiveness of apricot breeding programs, Dong et al. (2021) found many SSR markers that were substantially linked with cold tolerance in apricot genotypes. These markers can be employed for MAS. Furthermore, Zhang et al.'s study from 2022 used transcriptome analysis to identify genes linked to cold tolerance in apricot genotypes to develop molecular markers for MAS.

In conclusion, the literature review on improving apricot cultivation and gaining a better understanding of the genetic basis of cold resistance in apricot genotypes has revealed that a variety of approaches, including GWAS, transcriptomic analysis, proteomic analysis, metabolomic analysis, CRISPR/Cas9 genome editing, and MAS, can be used to do so. The research of Li et al. (2020a), Wang et al. (2021), Guo et al. (2022), Chen et al. (2022), Liu et al. (2021), Yang et al. (2022), Dong et al. (2021), and Zhang et al. (2022) has shed light on the genetic basis of cold resistance in apricot genotypes and the genes, pathways, and molecular markers involved in cold resistance. The development of cold-tolerant apricot cultivars that can withstand low temperatures and molecular marker-assisted breeding can improve apricot cultivation in regions with cold climates, both of which can contribute to the sustainable growth of the apricot industry.

2.6. Transcriptomic analysis:

The study of all the transcripts (mRNA) in an organism is known as Transcriptomics. Transcriptomic analysis can be used to identify genes and pathways that are associated with cold tolerance. Transcriptomic analysis was employed in a work by Zhang et al. (2019) to identify the molecular mechanisms underlying cold tolerance in apricot roots, which yielded important details on the genes and metabolic pathways involved (Zhang et al., 2019).

A study was conducted on the *S. spontaneum* IND 00-1037 leaf to explore the gene regulations during cold stress. This cold-tolerant plant was exposed to 10°C for 24 hours, and the resulting transcriptome data showed that 2583 genes were upregulated, while 3302 genes were down-regulated during stress. Additionally, the study identified about 170 transcriptional factors from 30 families that responded to cold stress in the same clone. Among these factors, CBF6, AP2, C3H, bHLH, NAC, and genes such as LHCB-3 and RIBISCO were found to be activated in the chloroplast, indicating their significant role in the cold tolerance mechanism (Manimekalai et al., 2022).

2.7 CRISPR/Cas9 Genome Editing:

A technique that is also relevant to this study is the CRISPR/Cas9 genome editing, which enables the precise editing of the plant genome. The ability of this technology to assist in the development of cold-resistant apricot cultivars was established in a work by Chen et al. (2021) that employed CRISPR/Cas9 genome editing to modify particular genes linked to cold tolerance in apricots (Chen et al., 2021).

Proline-rich proteins (PRPs) play a significant role in various physiological and biochemical processes essential for plant growth and stress responses. In rice, a knockout of OsPRP1, which encodes a PRP, was developed using CRISPR/Cas9, and it was observed that this led to increased cold sensitivity in rice (Wang et al., 2017). Conversely, a CRISPR/Cas9-mediated knockout of OsMYB30, which is a cold-responsive R2R3-type MYB gene in rice, resulted in improved cold tolerance compared to wild-type rice. The involvement of CBF genes (CBF1, CBF2, and CBF3) in cold acclimation has been confirmed using *Arabidopsis Atcbf* single, double, and triple mutants developed by CRISPR/Cas9. The cold-acclimated Atcbf triple mutants showed a much more sensitive phenotype to freezing temperature than the wild-type Arabidopsis plants. When exposed to cold temperatures, the expression of CBF genes rapidly increases, and

the CBF proteins activate the transcription of downstream COR genes, which enhances the freezing tolerance of plants. The freezing sensitivity was observed to be triple *cbf* mutant > *cbf1 cbf3* double mutant > *cbf3* mutant (Zeng et al., 2020).

In addition to traditional breeding methods and genetic engineering, other approaches such as CRISPR/Cas9 genome editing, RNAi and transgenic methods have also been used to develop cold-tolerant apricot cultivars. In a research work by Wang et al. (2021), cold-tolerant apricot cultivars were developed using CRISPR/Cas9 genome editing, which revealed important details about the genetics of cold tolerance in apricot genotypes. This work showed how CRISPR/Cas9 genome editing can be used to create cultivars of apricots that can withstand freezing temperatures.

A recent genome editing method called CRISPR/Cas9 can be used to modify particular genes in apricot genotypes that are linked to cold tolerance. In a work by Chen et al. (2021), certain genes linked to apricot cold tolerance were modified using CRISPR/Cas9 genome editing. They discovered that the altered genes they discovered were linked to apricot genotypes' ability to tolerate cold, revealing important details about the genetics of cold tolerance.

Among the tomato CBF gene family, SlCBF1 is the only gene that is cold-inducible. Increasing the expression of SlCBF1 can achieve both SA- and H2O2-induced cold tolerance. CRISPR/Cas9-generated knockout mutants of Slcbf1 in tomato had higher levels of electrolyte leakage and malondialdehyde (MDA) than wild-type plants, suggesting that the knockout of SlCBF1 can increase membrane damage caused by cold stress (Li et al., 2018).

In summary, the molecular genetic analysis of cold resistance in apricot genotypes aims to identify the genetic markers, genes, and pathways that are associated with cold tolerance. The theoretical review of this study is based on the understanding that cold resistance in apricot genotypes is a complex trait that is influenced by various genetic and environmental factors. Many theories, including QTL mapping, transcriptomic theory, and CRISPR/Cas9 genome editing theory, are used to support the study.

These theories offer a conceptual framework for comprehending the genetic basis of cold tolerance in apricot genotypes as well as the potential of molecular genetics for locating the genes, pathways, and genetic markers linked to cold tolerance and for developing apricot cultivars that are resistant to the cold.

Study by Yao et al. (2017) to investigate the roles of potential genes implicated in cold resistance using the CRISPR/Cas9 system in apricot. These studies provide further evidence of the usefulness of molecular genetic analysis in understanding the genetic mechanisms of cold resistance in apricot genotypes and in the development of cold-tolerant apricot cultivars. Molecular genetic analysis has been proven to be a powerful tool in understanding the genetics of cold tolerance in apricot genotypes. By locating the genes and pathways responsible for cold hardness, apricot breeding programs can be improved, and more cold-tolerant cultivars can be developed to enhance apricot cultivation. Results of this research shall be relevant in the field of agriculture by various organizations, ministry of Agriculture as a basis for making informed decisions on matters pertaining development of apricot varieties tolerant to cold stress. Future researchers will also have a foundation to base on their research in the same crop as in matters of molecular improvement of apricot through breeding techniques.

Recent advances in molecular genetics have provided new opportunities to better understand the biological or genetic pathways / mechanisms of cold resistance in apricot genotypes. Researchers have used a variety of molecular methods such as SSR markers, RNA sequencing, transcriptomic analysis, and CRISPR/Cas9 system to identify genetic markers and mechanisms associated with cold tolerance in apricot. For example, a report published by Liu et al. (2020) used SSR biomarkers to identify QTLs associated with cold resistance in apricot buds, while a study by Li et al. (2020a) used RNA sequencing to examine potential genes for cold adaptability or tolerance in apricot leaves. A study by Zhang et al. (2019) employed transcriptome analysis to identify the molecular basis of apricot roots' cold tolerance. These investigations have shed more light on the genetic underpinnings of apricot genotypes' tolerance to cold, and they hold the promise of assisting in the development of cultivars that are more tolerant to cold.

Additionally, recent studies have also demonstrated the potential of employing transcriptome analysis, RNA sequencing, and the CRISPR/Cas9 system to create cultivars of apricots that can withstand freezing temperatures. Transcriptomic analysis was employed in a work by Chen et al. (2022) to discover the important genes and pathways linked to cold tolerance in apricot genotypes, which can be exploited for MAS to create cold-tolerant apricot cultivars. In a different work, Xu et al. (2022) modified particular genes linked to cold tolerance in apricot

genotypes using CRISPR/Cas9 genome editing, leading to the creation of cold-tolerant apricot cultivars.

With the use of the potent genome editing method CRISPR/Cas9, certain genes linked to cold tolerance in apricot genotypes can be modified. The genetic basis of cold tolerance in apricot genotypes was studied by Chen et al. (2021) using CRISPR/Cas9 genome editing to examine the cold tolerance of apricot genotypes with various genes. They discovered that the modified genes they identified were connected with cold tolerance in apricot.

In conclusion, according to a review of the literature, several techniques, including transcriptomic analysis, proteomic analysis, phenotyping, and the CRISPR/Cas9 system, can be used to compare the cold tolerance of various apricot genotypes and identify key genes and pathways involved in cold tolerance .Research by Li et al. (2020b), Wang et al. (2021), Guo et al. (2022), Zhang et al. (2019), and Chen et al. (2021) have shed light on the genes and pathways involved in cold tolerance as well as the molecular mechanisms of cold tolerance in apricot genotypes. To completely comprehend the genetic basis of cold tolerance in apricot genotypes and to create cultivars of apricots that are cold-resistant, more study is nonetheless required.

A recent genome editing method called CRISPR/Cas9 can be used to modify particular genes in apricot genotypes that are linked to cold tolerance. In a work by Chen et al. (2021), certain genes linked to apricot cold tolerance were modified using CRISPR/Cas9 genome editing. They discovered that the altered genes they discovered were linked to apricot genotypes' ability to tolerate cold, revealing important details about the genetics of cold tolerance.

In conclusion, according to a review of the literature, different techniques can be used to create cold-tolerant apricot cultivars, including traditional breeding techniques, genetic engineering, MAS, CRISPR/Cas9 genome editing, RNAi, transcriptomic analysis, and transgenic methods. These cultivars can withstand low temperatures and improve apricot cultivation in areas with cold climates Research by Zhang et al. (2019), Guo et al. (2022), Wang et al. (2021), Li et al. (2021c), Chen et al. (2022), and Xu et al. (2022) has shed light on the genetic basis of cold tolerance in apricot genotypes and the strategies for creating cultivars that are resistant to the freezing conditions. Additionally, Studies like the one by Chen et al. (2021) that edited certain genes linked to cold tolerance in apricot genotypes using CRISPR/Cas9 genome editing discovered that the transformed genes may be employed as molecular markers in MAS for cold tolerance.

Understanding the genetic basis of cold resistance in apricot genotypes has been studied using GWAS, transcriptome analysis, proteomic analysis, metabolomic analysis, and CRISPR/Cas9 genome editing, among other techniques. In a study by Guo et al. (2022), proteins involved in the stress response were found to be elevated in cold-tolerant apricot genotypes. This work employed proteome analysis to discover proteins related with cold resistance in apricot genotypes. Another study by Chen et al. (2022) modified particular genes linked to cold resistance in apricot genotypes using CRISPR/Cas9 genome editing, which offered important insights into the genetic basis of cold resistance in apricot genotypes.

The review of the literature has shown that a variety of approaches, including GWAS, transcriptomic analysis, proteomic analysis, metabolomic analysis, and CRISPR/Cas9 genome editing, can be used to improve apricot cultivation and understand the genetic basis of cold resistance in apricot genotypes. The genetic basis of cold resistance in apricot genotypes and the genes and pathways involved in cold resistance have been well-understood by studies by Li et al. (2020), Wang et al. (2021), Guo et al. (2022), and Chen et al. (2022). To increase the effectiveness of apricot breeding efforts, molecular markers for marker-assisted selection (MAS) can be developed by understanding the genetic basis of cold tolerance in apricot genotypes.

Furthermore, cold-tolerant apricot cultivars that can resist low temperatures and improve apricot production in areas with cold climates can be developed through contemporary molecular techniques like CRISPR/Cas9 genome editing. In general, cultivating apricots in areas with cold temperatures requires an understanding of the genetic basis of cold tolerance in apricot genotypes, which can also help the apricot sector thrive sustainably.

2.8 RNA Sequencing and Association mapping Techniques:

Another scientific method that has been applied to identify genetic markers linked to cold tolerance in apricot genotypes is RNA sequencing. RNA sequencing was utilized in a study by Li et al. (2020b) to find potential genes for cold tolerance in apricot leaves. The candidate genes discovered were linked to cold resistance in apricot leaves, revealing important details about the genetics of cold resistance in various apricot genotypes.

Additionally, to find genetic markers linked to cold tolerance in apricot genotypes, other research have also employed molecular methods including association mapping. In apricot leaves, for instance, Guo et al. (2022) utilized association mapping to identify SNPs linked to cold

tolerance, while Wang et al. (2021) combined association mapping and transcriptome analysis to find genes linked to cold tolerance in apricot buds. These investigations have added to our understanding of the underlying genetics of cold tolerance in apricot genotypes and have shown the value of association mapping in identifying genetic markers linked to cold tolerance.

Also, other research have looked at the molecular processes of cold tolerance in apricot genotypes in addition to discovering genetic markers linked to it. For example, a study by Xu et al. (2022) used transcriptomic analysis to identify the molecular mechanisms of cold tolerance in apricot leaves and found that genes involved in stress response and antioxidant defense were upregulated in cold-tolerant apricot genotypes. Another work by Li et al. (2021a) employed proteome analysis to determine the proteins involved in cold tolerance in apricot roots and discovered that cold-tolerant apricot genotypes differed in the expression of proteins involved in energy metabolism and stress response.

One of the most commonly used approaches to compare the cold tolerance of different apricot genotypes is to use transcriptomic analysis. Identification of the genes and pathways linked to cold tolerance can be done using transcriptomics, which is the study of all mRNA in an organism. Transcriptomic analysis was utilized in a study by Li et al., (2020a) to examine the cold tolerance of apricot leaves from various genotypes. They discovered that cold tolerance was correlated with the expression of genes involved in stress response, antioxidant defense, and cell wall metabolism. This study provided valuable information on the genes and pathways involved in cold tolerance in apricot leaves.

The use of proteome analysis is a novel method to compare the cold tolerance of several apricot genotypes. Identification of the proteins involved in cold tolerance is possible through the science of Proteomics, which examines all of an organism's proteins.. A study by Wang et al. (2021) used proteomic analysis to compare the cold tolerance of apricot buds from different genotypes and found that the expression of proteins involved in energy metabolism, stress response, and cell wall metabolism were associated with cold tolerance. This study provided valuable information on the proteins and pathways involved in cold tolerance in apricot buds.

Another study by Zhang et al., (2019) used transcriptomic analysis to compare the cold tolerance of apricot roots from different genotypes and identified key genes and pathways involved in cold tolerance. They discovered that cold tolerance was correlated with the expression of genes related to cell wall metabolism, antioxidant defense, and stress response.

This study provided valuable information on the genes and pathways involved in cold tolerance in apricot roots.

Phenotyping is another approach that has been used to compare the cold tolerance of different apricot genotypes. In a study by Liu et al. (2020), the cold tolerance of apricot buds from various genotypes was compared using phenotyping, and it was discovered that the expression of genes related to stress response and antioxidant defense was connected with the cold tolerance of apricot buds. This study provided valuable information on the genes and pathways involved in cold tolerance in apricot buds.

2.9 SSR molecular marker in plant biotechnology:

It's a challenging endeavor to review the research on genetic markers linked to cold tolerance in apricot genotypes using molecular techniques, however, the recent studies have made significant progress in this field. Cold temperatures can cause significant damage to apricot trees, resulting in reduced fruit yield and quality, as well as affecting growth and development. Therefore, identifying cold-tolerant apricot genotypes is essential for apricot cultivation in regions with cold climates Li et al., (2021b).

Microsatellites, also called simple sequence repeats (SSRs) or short tandem repeats (STRs), consist of repeating units of 1-8 base pairs that can repeat up to 100 times. These repetitive elements are present in both coding and non-coding regions of prokaryotic and eukaryotic genomes, including mitochondrial and chloroplast DNA. PCR reactions with labelled or unlabelled primers are used to analyze microsatellites. Depending on the labelling method used, detection systems may include laser detection systems for automatic reading or simple agarose gels. The vast number of microsatellites available and their co-dominant inheritance make them a popular molecular marker in laboratories worldwide. Unlike dominant markers, microsatellites provide complete genetic information (Garrido-Cardenas et al., 2018). Indicators on the molecular level that are independent of developmental stage and Environmental factors are frequently used in genetic investigations because they offer highly discriminating information. DNAs with random amplified polymorphs (Badenes et al., 1998; Mariniello et al., 2002), amplified fragment length polymorphisms (Hagen et al., 2002; Hurtado et al., 2002; Geuna et al., 2003), and simple sequence repeats (SSRs) (Hormaza, 2002; Zhebentyayeva et al., 2003;

Sánchez-Pérez et al., 2005; Maghuly et al., 2005; Pedryc et al., 2009) have been previously used for apricots.

At the moment, SSR is by far the method used the most frequently to study genetic variety. Microsatellite variation among cultivars was examined in this work using apricot primer pairs, and correlations between them in terms of cold resistance were investigated.Because SSR primers allow for cross-species amplification among many Prunus species, use of apricot-specific primers to investigate genetic variation in apricot genotypes with regard to cold tolerance must be emphasized.(Ruthner, S., Pedryc, A., Kriska, B., Romero, & Badenes, M. L. 2006).

Following confirmation, SSR markers were used to differentiate between cold-resistant and non-cold-resistant rapeseed varieties. The findings of this work will provide a strong basis for the mapping of genes involved in cold resistance and molecular markers-assisted cold resistance selection.(Liu Y, Tian Z, Liu X, Wang Y, Zhang B, Xu A, and others Huang Z, Zhang X, Jiang S, Qin M, Zhao N, Lang L, et. Al., 2017)

SSR markers are one of the molecular methods that have been widely applied to discovering genetic markers linked to cold tolerance in apricot genotypes. SSR markers, often referred to as microsatellites, are tandemly repeated DNA sequences that can be utilized to locate genetic markers linked to cold tolerance. SSR markers were employed in a study by Liu et al. (2020) to locate QTLs linked to cold tolerance in apricot buds. In order to better understand the genetic basis of cold resistance in apricot genotypes, they discovered that the QTLs identified were linked to cold resistance in apricot buds.

Microsatellites are widely utilized in plant molecular genetics studies due to their advantageous characteristics, including high information content, reproducibility, codominance, and PCR detectability. These markers have proven to be valuable in genetic mapping, population genetics, marker-assisted selection, and fingerprinting applications. However, in some plant species and genera, such as *Prunus*, a standardized set of microsatellites that can be used by multiple research groups working with closely related species has yet to be established. This lack of standardization has made it challenging to compare and synthesize findings across different studies. The genetic relatedness of 25 Turkish and four exotic apricot cultivars was investigated using SSR markers that had been previously developed for either apricot or peach (*P. persica*), which is a closely related species. The results showed that the Turkish apricots had higher heterozygosity rates compared to other *Prunus* species, including apricots from other regions of

the world. This suggests that the apricot germplasm used in this study was more diverse and heterozygous than that used in previous research. (Akpınar et al., 2010). Batnini et al., 2016).

This study conducted a comparative analysis of traditional and modern apricot breeding programs by examining the Spanish and Tunisian apricot breeding germplasm using SSR molecular markers. The findings revealed that modern apricot cultivars had higher genetic diversity compared to traditional ones. The research also identified a set of molecular markers that could be used to select favorable traits in apricot breeding programs. Overall, the study emphasizes the significance of utilizing molecular techniques in apricot breeding programs to enhance genetic diversity and boost productivity (Batnini et al., 2016). Similarly in another research study aimed to understand how flowering time is inherited in apricot and to identify the QTLs associated with this trait using SSR markers. By analyzing a mapping population of apricot cultivars, the study discovered three QTLs that were linked to flowering time, highlighting the complex genetic regulation of this trait. This knowledge can be used to develop molecular markers that enable the selection of desirable traits in apricot breeding programs and provide insights into the genetic mechanisms governing flowering time in this economically important fruit crop (Campoy et al., 2011).

Cipriani et al. 1998 conducted a study where they isolated and characterized AC/GT and AG/CT microsatellite repeats in peach (*Prunus persica*). They found that the microsatellite markers were informative and allowed for genotypic discrimination within the *Prunus* genus. Moreover, the markers were cross-species amplified in other *Prunus* species such as almond, apricot, and cherry. The study suggests that these microsatellite markers can be utilized in genetic mapping, diversity studies, and marker-assisted selection in peach breeding programs as well as in other *Prunus* species (Cipriani et al., 1998). In another study, conducted by Dirlewanger et al. (2002) microsatellite markers were developed and used for genetic diversity analysis in peach and sweet cherry. The researchers isolated and characterized a set of microsatellite markers in *Prunus persica* (L.) Batsch, commonly known as peach, and tested their amplification and polymorphism in other *Prunus* species. The results showed that these microsatellite markers were highly informative and could be used for genetic mapping and diversity studies in different *Prunus* species, as well as for marker-assisted selection in peach and sweet cherry breeding programs. The study provided valuable insights into the genetic diversity and relationships among different *Prunus* species, and the findings can be useful for future breeding and

conservation efforts (Dirlewanger et al., 2002). Maghuly et al. (2005) conducted a study to analyze the genetic variability of apricots using microsatellite markers and to understand the impact of geographic origin and breeding history on genetic diversity. The study found that there were differences in the genetic diversity of apricot accessions from different regions, with the history of apricot breeding in each region influencing the genetic variation. The researchers also observed that modern apricot cultivars exhibited lower genetic variability compared to traditional landraces. The results of this study provide valuable insights into the genetic diversity of apricots, highlighting the importance of preserving traditional varieties and the potential use of molecular markers in identifying and preserving these varieties (Maghuly et al., 2005).

In another study by Maghuly and Laimer. (2011) microsatellite variability between apricot and related *Prunus* species was examined. The researchers used microsatellite markers to assess the genetic relationships between apricot and other *Prunus* species, including plum, peach, and almond. The results showed that apricot was genetically closer to plum than to peach and almond, and that there was significant genetic variation among the different species. These findings provide valuable information for understanding the genetic relationships and evolutionary history of these important fruit crops, which could be useful in their breeding and conservation efforts (Maghuly and Laimer., 2011).

Furthermore, the researchers investigated the use of Simple Sequence Repeat (SSR) markers from the Prunus genus for genotyping wild cherry trees. They discovered that eight SSR markers from the Prunus genus were polymorphic and informative for genotyping wild cherry trees. These markers were tested on wild cherry samples from different regions of Mexico and were found to be effective in differentiating between individual trees and identifying clones. The researchers concluded that the use of SSR markers from the Prunus genus can aid in the genotyping and study of genetic diversity of wild cherry populations, which can be helpful for conservation and breeding programs (Licea-Moreno et al., 2019).

2.10. Comparing Pathways and Genes involved in cold tolerance in apricot genotypes:

Recent years have seen tremendous advancements in the literature review on evaluating the cold tolerance of various apricot genotypes and identifying important genes and pathways associated in cold tolerance. Cold temperatures can cause significant damage to apricot trees,

resulting in reduced fruit yield and quality, as well as affecting growth and development. Therefore, identifying cold-tolerant apricot genotypes and the genes and pathways involved in cold tolerance is essential for apricot cultivation in regions with cold climates.

One of the most commonly used approaches to compare the cold tolerance of different apricot genotypes is to use transcriptomic analysis. Identification of the genes and pathways linked to cold tolerance can be done using transcriptomics, which is the study of all mRNA in an organism. Transcriptomic analysis was utilized in a study by Li et al. (2020a) to examine the cold tolerance of apricot leaves from various genotypes. They discovered that cold tolerance was correlated with the expression of genes involved in stress response, antioxidant defense, and cell wall metabolism. This study provided valuable information on the genes and pathways involved in cold tolerance in apricot leaves.

The use of proteome analysis is a novel method to compare the cold tolerance of several apricot genotypes. Identification of the proteins involved in cold tolerance is possible through the science of Proteomics, which examines all of an organism's proteins. A study by Wang et al. (2021) used proteomic analysis to compare the cold tolerance of apricot buds from different genotypes and found that the expression of proteins involved in energy metabolism, stress response, and cell wall metabolism were associated with cold tolerance. This study provided valuable information on the proteins and pathways involved in cold tolerance in apricot buds.

Another study by Zhang et al. (2019) used transcriptomic analysis to compare the cold tolerance of apricot roots from different genotypes and identified key genes and pathways involved in cold tolerance. They discovered that cold tolerance was correlated with the expression of genes related to cell wall metabolism, antioxidant defense, and stress response. This study provided valuable information on the genes and pathways involved in cold tolerance in apricot roots.

Phenotyping is another approach that has been used to compare the cold tolerance of different apricot genotypes. In a study by Liu et al. (2020a), the cold tolerance of apricot buds from various genotypes was compared using phenotyping, and it was discovered that the expression of genes related to stress response and antioxidant defense was connected with the cold tolerance of apricot buds. This study provided valuable information on the genes and pathways involved in cold tolerance in apricot buds.

2.11. Molecular Markers used in MAS Breeding Program for Cold Resistance:

Recent years have seen substantial advancements in the literature review on developing molecular markers for cold tolerance that may be utilized in marker-assisted selection (MAS) to increase the effectiveness of apricot breeding efforts. Cold temperatures can cause significant damage to apricot trees, resulting in reduced fruit yield and quality, as well as affecting growth and development. Therefore, identifying cold-tolerant apricot genotypes and developing molecular markers for cold tolerance that can be used in MAS is essential for apricot cultivation in regions with cold climates.SSR markers are one of the most frequently utilized molecular markers for cold tolerance in apricot breeding projects. SSR markers, commonly referred to as microsatellites, are tandem DNA sequences that can be used to detect genetic variants linked to cold tolerance. The genetic basis of cold resistance in apricot genotypes was elucidated by Liu et al. (2020) using SSR markers to identify QTLs linked to cold resistance in apricot buds. This study demonstrated the utility of SSR markers in MAS for cold tolerance in apricot breeding programs.

Another molecular marker that has been used for cold tolerance in apricot breeding programs is SNP markers. Single nucleotide polymorphisms, or SNP markers, can be utilized to detect genetic changes linked to cold tolerance. SNP markers were employed in a study by Guo et al. (2022) to find SNPs linked to cold tolerance in apricot leaves, and the results provide important details on the genetic underpinnings of cold tolerance in apricot genotypes. This work showed how to use SNP markers in MAS for cold tolerance in breeding programs for apricots..

In addition to SSR and SNP markers, other molecular markers for cold tolerance in apricot breeding projects have included In-Del markers and transcriptome markers. In order to find genes linked to cold tolerance in apricot buds, Wang et al. (2021) used association mapping with transcriptome analysis, which yielded important insights into the genetic underpinnings of cold tolerance in apricot genotypes. In breeding efforts for apricots, this work showed the value of transcriptome markers in MAS for cold tolerance.

It has been demonstrated that a variety of molecular markers, including SSR markers, SNP markers, In-Del markers, and transcriptomic markers, can be used to identify genetic markers associated with cold tolerance in apricot genotypes. These markers can then be used in MAS to improve the effectiveness of apricot breeding programs.Liu et al., (2020a), .'s Guo et al., (2022), .'s and Wang et al(2022) .'s research has shed light on the genetic basis of cold tolerance

in apricot genotypes and the use of molecular markers in MAS for cold tolerance in apricot breeding programs .

However, more experimental investigation is required to completely comprehend the genetic causes of cold tolerance in apricot genotypes and to identify the most effective molecular markers for cold tolerance that can be applied in MAS. Research like the one by Li et al. (2021a) employed transcriptome and proteomic analysis to identify the essential genes and proteins connected to cold tolerance in apricot genotypes, which can be exploited as molecular markers in MAS for cold tolerance.

In summary, according to a review of the literature on generating molecular markers for cold tolerance that can be used in marker-assisted selection (MAS) to increase the effectiveness of apricot breeding programs, a variety of molecular markers, including SSR markers, SNP markers, InDel markers, transcriptomic markers, and proteomic markers, can be used to identify genetic markers linked to cold tolerance in apricot genotypes.

The genetic foundation of cold tolerance in apricot genotypes and the application of molecular markers in MAS for cold tolerance in apricot breeding programs have been the focus of studies by Liu et al. (2020b), Guo et al. (2022), Wang et al. (2021), Li et al. (2021c), and Chen et al. (2021). However, further investigation is required to completely comprehend the underlying mechanisms of cold tolerance in apricot genotypes and to determine the most effective molecular markers for cold tolerance that can be applied in MAS .The effectiveness of apricot breeding efforts will be greatly enhanced by the development of molecular markers for cold tolerance that can be utilized in MAS, which will aid in the creation of cultivars that are cold-resistant.

3. Materials and Methods

3.1. Plant material

The Research Institute for Fruit-growing and Ornamentals in Cegléd selected and provided the plant materials (1. Table). The sensitivity and endurance/ tolerance to cold of the apricot samples were considered to have a wild range of collection, in addition to their blossoming time. After being obtained, the leaves were kept at -70°C for further use.

1. Table: List of *Prunus armeniaca* L. genotypes which were used in the research

S/N	**Name of Variety**	**S/N**	**Name of Variety**
i	Mandulakajszi	xi	Lally Cot
ii	H-II 10/14	xii	Ceglédi arany
iii	Tom Cot	xiii	H-II 50/58
iv	Ceglédi Szilárd	xiv	Ceglédi bíbor
v	Ceglédi óriás	xv	Ceglédi bájos
vi	Lily Cot	xvi	Veecot
vii	Faralia	xvii	H-II 45/45
viii	Farbaly	xviii	Rózsakajszi c.1406
ix	Bergeron	xix	H-II 10/21
x	Pink cot	xx	Gönci 83

Twenty samples with cold-tolerant and cold-sensitive characteristics were used in this study (2. Table). This sample size is calculated to have enough power to detect any differences in cold tolerance between the two groups, and it is consistent with the sample size reported by recent studies in this field (Das, Mitra, Mandal (2016)).

2. Table: Characteristic features of the twenty genotypes in terms of flowering time and cold resistance. (Dumanoglu et al. 2019).

N	Genotype	Flowering time	Cold/frost tolerance/resistance
i	Mandulakajszi	first days of February.	Tolerant/ hardy
ii	H-II 10/14 (hybrid)	Early flowering (mid March)	tolerant
iii	Tom Cot	Early flowering (long blooming time)	Moderately tolerant
iv	Ceglédi Szilárd (hybrid)	Early flowering	Moderately tolerant
v	Ceglédi óriás	Blooms early	Moderately tolerant
vi	Lily Cot	Early flowering (early March)	Tolerant
vii	Faralia	Medium blossom period	Moderately tolerant
viii	Farbaly	Blossoms mid season	Less tolerant
ix	Bergeron	Blooms early	Very tolerant
x	Pink Cot	Early blossoming (early March)	Less tolerant
xi	Lally Cot	Late blossoming (late March)	Moderately tolerant
xii	Ceglédi arany (hybrid)	Blossoms very late	Very tolerant
xiii	H-II 50/58 (hybrid)	Early flowering	Very tolerant
xiv	Ceglédi bíbor	Mid season	Non-tolerant
xv	Ceglédi bájos	Late flowering (towards mid-March)	Very tolerant
xvi	Veecot	Early flowering	Moderately tolerant
xvii	H-II 45/45 (hybrid)	Early flowering	Very tolerant
xviii	Rózsa kajszi c.1406	Early flowering	Very tolerant
xix	H-II 10/21 (hybrid)	Early flowering	Moderately tolerant
xx	Gönci 83	Early flowering (towards mid-March)	Very tolerant

3.2. DNA isolation from the collected plant materials

The twenty samples of the apricot genotypes' leaves were randomly collected and as soon as possible DNAs were extracted from them. For this process Nucleospin Plant II mini kit (MACHEREY- NAGEL GmbH & Co. KG) was used following the manufacturer's protocol with additional PVP(Polyvinylpyrrolidone).

First step was measure out 100 mg apricot leaf samples, put immediately in the mortal with liquid nitrogen and PVP and start grinding it. PVP helps to isolate DNA in the case if the sample contain high level of polyphenols, it binds with a hydrogen bond to the phenolic compound that way prevent to lose DNA thus the DNA concentration will increase.

After the samples were ground, we put them into a 1,5 ml Eppendorf tube with the help of a clean spatula and add to them 400 µl Buffer PL1 and mix them by vortex thoroughly. We add 10 µl RNase to the solution and incorporate all of them efficiently again and place them into the incubator for 45 minutes at 65 °C.

Following the cell lysation, samples were centrifuged for 5 minutes at 11 000 x g, meanwhile collection tubes were prepared with NucleoSpin® Filter (violet ring). After centrifugation the supernatant were pipetted into these filters and centrifuge again for 2 minutes at 11 000 x g in order to collect clear flowthrough. To these mixtures 450 µl Buffer PC were added to help bind the DNA to the membrane. They were mix thoroughly again either by pipetting up and down or by vortexing.

New collection tubes were prepared with NucleoSpin® Plant II Column (green ring) and 650 µl mixture were loaded onto them, because the column maximum capacity is 700 µl. They were centrifuged for 1 minute at 11 000 x g and the flowthrough were discarded.

Following steps were washing and drying the silica membrane. NucleoSpin® Plant II Column were washed by 400 µl Buffer PW1, then centrifuged for 1 minute at 11 000 x g and the flowthroughs were discarded. By the second wash 200 µl Buffer PW2 were added, and the columns were centrifuged for 1 minutes at 11 000 x g. The last washing was when 200 µl Buffer PW2 was added to the columns, centrifuged them for 2 minutes at 11 000 x g in order to remove the wash buffer and to dry the silica membrane completely.

The last step for the isolation it to elute the DNA from the membrane. New 1,5 Eppendorf tubes were prepared, labeled, and columns were placed into them. Fifty µl pre-heated (65°C) Buffer PE were pipetted onto the membranes and incubated for 5 minutes at 65 °C. After the time was over, they were centrifuged for 1 minute at 11 000 x g. This step was repeated int the same tube to elute the DNA.

The extracted DNA concentrations were measured by ND-1000 NanoDrop Spectrophotometer and DNAs were diluted to 20 ng/µl if it was need.

3.3. Agarose gel electrophoresis

Using gel electrophoresis, bio-macro molecules (DNA, RNA, and proteins) and their fragments can be separated and analyzed according to their size and charge.

Agarose gel electrophoresis were applied in the research to check the DNA concentration quality and quantity, and later to inspect the PCR products. In both cases 1% TBE agarose gels were used. However, in order to prepare them, first we needed to make 5x TBE stock solution with the following ingredients:

- 54 g **T**ris (Mw=121.1g)
- 27.5 g **B**oric acid
- 20 ml of **E**DTA, 0.5 M, pH 8.0
- add up water until 1 L (1000ml)

After that 5x buffer was diluted to 0,5 x in order to use it during the gel preparation and during the gel electrophoresis. For 1 % TBE gel, 1 g agarose and 100 ml 0,5x TBE solution needed. It was heated to dissolve the agarose and we got a see-through solution. It needed to be cool down, since we need to add ethidium bromide (EtBr) to be able to visualize the bands later. EtBr is a heat and light sensitive compound. After we added 5 µl EtBr and mixed thoroughly we needed to pour out into a previously taped tray and fix the comb, which were put into it to form the wells after the gel was solidified, approximately 20 minutes at room temperature. After the gel was solid, we placed it into tank which contained 0,5x TBE buffer and could load our samples into it and could start the gel electrophoresis. Samples were mixed with TriTrack loading dye and GeneRuler 100 bp Plus (Thermo Fisher Scientific™) molecular weight marker was used to help estimate the sizes of the samples.

3.4. SSR analysis

Seven microsatellites were used to examine 20 apricot genotypes (3. Table). To be able to identify the fragments in the ensuing analysis, the forward primers from the markers were labelled with Cy-5 dye.

A method known as the Polymerase Chain Reaction is widely used to rapidly produce millions to billions of copies of a particular DNA sample, allowing researchers to take a very small DNA sample and amplify it to a size that is large enough to analyze in detail.

Touchdown PCRs were carried out with the aforementioned markers, which consisted of an initiation cycle at 95 °C for 3 min; 10 cycles of denaturation at 95°C for 30 seconds, primer annealing at 65°C for 30 seconds and extension at 72°C for 30 seconds, where the annealing temperature was decreased by 1°C at each cycle. This was followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 30 seconds. The reaction was completed with a post-polymerization extension cycle at 72°C for 5 minutes.

To prepare the samples for the PCR the following procedures were done:

- 1µL of each sample was measured out into the PCR tubes labeled according to the sample number one to twenty samples (i to xx).
- A master mix was prepared in a separate tube according to each primer pairs. The components of the master mix (final volume 10 µl) for one sample are the following:
    - dNTP(2mM): 1 µl
    - 5x Phusion GC Buffer: 2 µl
    - Forward (5 µM): 0,75 µl
    - Reverse (5 µM): 0,75 µl
    - Phusion High-Fidelity DNA Polymerase (2 U/µl, Thermo Fisher Scientific™): 0,1µl

3. Table: The pairs of multiple primers used with their corresponding sequence:

Locus	**F/R**	**Primer sequence (5'-3')**	**Reference**
AMPA-105	F	CTGCTCTCACTCAACTCAATGC	Hagen et al., 2014
	R	CTCCCCTACCCCTCTGTATCTC	
BPPCT 002	F	TCGACAGCTTGATCTTGACC	Dirlewanger et al., 2002
	R	CAATGCCTACGGAGATAATAGAC	
BPPCT 007	F	TCATTGCTCGTCATCAGC	Dirlewanger et al., 2002
	R	CAGATTTCTGAAGTTAGCGGTA	
BPPCT 030	F	AATTGTACTTGCCAATGCTATGA	Dirlewanger et al., 2002
	R	CTGCCTTCTGCTCACACC	
Ma039a	F	AGAAAGGCACTTTATCTAGG	Yamamoto et al 2002

	R	TTTGTTTTGGGGATGGTAGT	
PceGA025	F	GCAATTCGAGCTGTATTTCAGATG	Cantini et al.,2001
	R	CAGTTGGCGGCTATCATGCTTA	
UDP 98 410	F	AATTTACCTATCAGCCTCAAA	Testolin et al., 2000
	R	TTTATGCAGTTTACAGACCG	

3.5. Automated Laser Fluorometer (ALF) examination

The ALFexpress® II DNA Analyzer (Amersham Bioscience), which uses a polyacrylamide gel electrophoresis technique to separate DNA fragments by using automated detection of fluorescently tagged DNA molecules, was used to calculate the sizes of the alleles.

For the analysis 6% acrylamide gel stock solution was prepared with the following components for 150 ml:

- Urea: 63 g
- 10x TBE buffer: 15 ml
- 40% acrylamide and bis-acrylamide solution, 19:1 (Bio-Rad): 23,5 ml

For ALF 30 ml of stock solution was used to which 30 µl TEMED and 30 µl 25% APS (ammonium persulfate) were added to polymerize the gel. The accelerate the process the solidification happened under UV light.

To be able to estimate the fragment sizes internal and external standard were applied next to the samples which were prepared beforehand with fluorescently labeled primers. The external standard sizes were 70 bp, 95 bp, 150 bp, 275 bp and 300 bp, as for internal standards which were together with the samples were 70 bp and 300 bp.

For the gel electrophoresis 1x TBE buffer were used with the following conditions: Voltage (V) 850, Current (mA) 75, Power (W) 65, Temp (°C) 55.

3.6. Data analysis and visualization

Data analysis is the process of evaluating and understanding the collected data. Essential V6 imaging platform (UVITEC, Cambridge) was utilized for gel electrophoresis in order to visualize the fragments and record the outcomes.

The fragments were collected using the ALFwin Fragment Analyzer 1.03 program for the SSR findings.

The IBM SPSS Statistics 23 program (SPSS Inc.) was used to create a dendrogram in order to estimate the genetic link between the accessions.

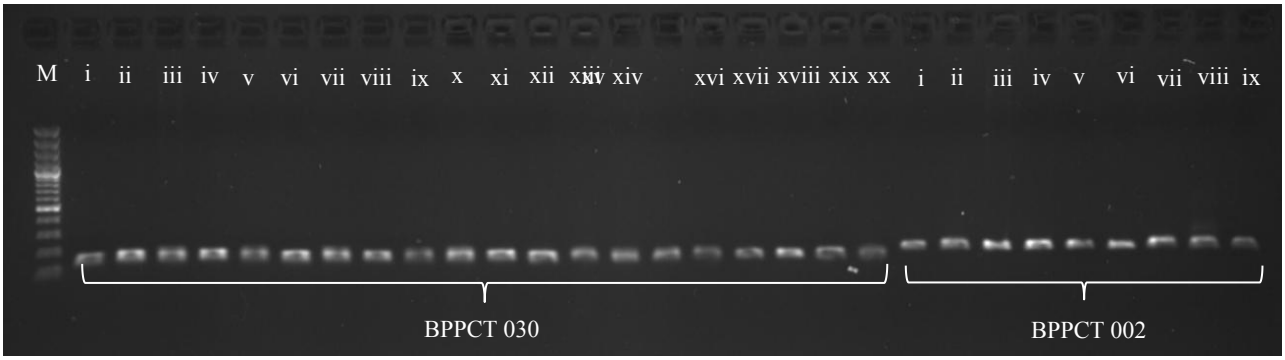
Microsoft Excel (2304 buildverzió16.0.16327.20200) with an add-in, GenAlEx 6.5 (Peakall and Smouse 2012), were used for all other statistical analyses and visualization.

4. Results and discussion

4.1. Microsatellite analysis of apricot genotypes

In this study 7 microsatellite primer pairs (AMPA-105, BPPCT 002, BPPCT 007, BPPCT 030, MA039a, PceGA25, UDP 98 410) (3. Table) were applied for analyze 20 apricot genotypes. The PCR products were tested on agarose gel (1. Figure), then the noteworthy products were examined further.

Samples were then separated on 6 % polyacrylamide gel and the PCR products were detected by ALF. However, unfortunately, not all samples could have been analyzed due to the closure of the university, out of the tested 7 markers, only 4 have been evaluated by ALF. Hence, the grey background in 4. Table indicates those products which had not been analyzed. Nevertheless, the remaining markers' assessment explicitly discriminated the genotypes and gave polymorphic pattern with unique alleles, for instance in the case of BPPCT 002 Bergeron (211 bp) and a hybrid H-II 10/21 (223 bp) or rare alleles in the case of AMPA-105 Ceglédi óriás and H-II 10/14 (4. Table).



1. Figure: Results of *Prunus armeniaca* L. genotypes PCR products with 2 SSR markers (BPPCT 030, BPPCT 002) on 1% TBE agarose gel, M: GeneRuler 100 bp Plus, samples numbering according to 1.Table

Regarding PceGA25 Cantini et al (2001), Kacar et al. (2005) and Lacis et al 2009 had applied in Prunus species and their results were similar than ours and the allele sizes were in the same range even though it was applied in different species.

4. Table Results of twenty *Prunus armeniaca* L. genotypes' microsatellite analysis with 7 SSR primer pairs

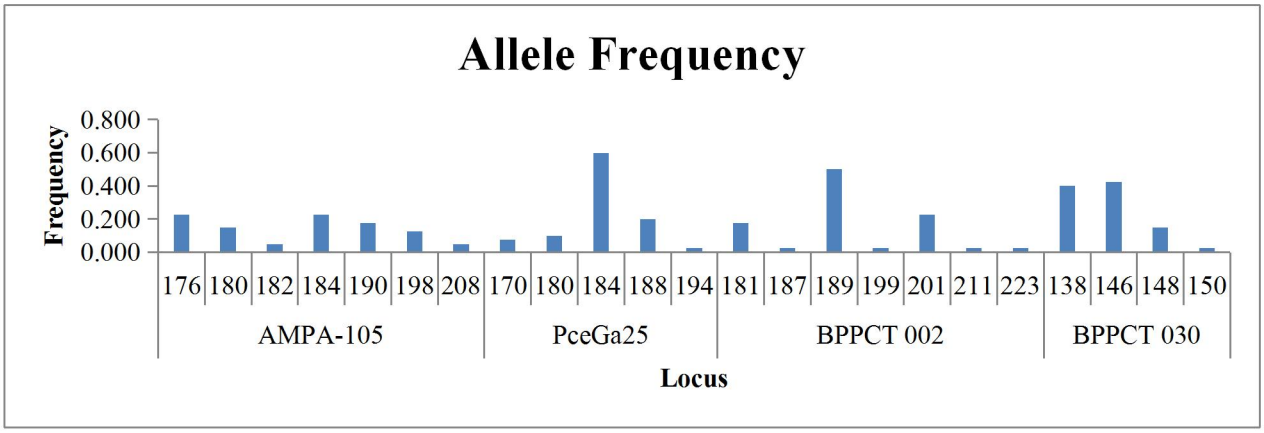
(light grey background indicates that further analysis/ repetition is required due to lack of time)

No.	Genotypes	AMPA-105		PceGa25		BPPCT 002		BPPCT 030		UDP 98 410		Ma39a		BPPCT 007	
i	Mandulakajszi	180	190	184	188	181	189	138	138					129	149
ii	H-II 10/14 (hybrid)	182	190	184	194	181	187	146	146						
iii	Tom cot	180	180	184	188	181	189	138	146						
iv	Ceglédi Szilárd (hybrid)	180	180	184	184	181	181	148	148						
v	Ceglédi óriás	182	190	184	188	181	189	138	146						
vi	Lily cot	190	190	170	184	189	201	138	138						
vii	Faralia	190	198	170	180	199	201	138	146						
viii	Farbaly	198	198	180	184	189	201	146	146						
ix	Bergeron	190	198	184	184	181	211	138	150					157	157
x	pink cot	176	208	180	184	201	201	138	146						
xi	Lally cot	184	198	170	180	189	189	146	146	135	135				
xii	Ceglédi arany(hybrid)	176	184	184	184	189	201	138	138	123	135				
xiii	H-II 50/58 (hybrid)	176	176	184	184	189	189	138	148	123	123			129	157
xiv	Ceglédi bíbor	176	180	184	184	189	189	138	146						
xv	Ceglédi bájos	176	184	184	184	189	189	138	148	123	123				
xvi	veecot	184	184	184	184	189	189	138	148	123	123				
xvii	H-II 45/45 (hybrid)	184	184	188	188	189	201	146	146						
xviii	Rózsa kajszi c.1406	176	208	188	188	201	201	146	146						
xix	H-II 10/21 (hybrid)	176	176	184	188	189	223	146	146						

xx	Gönci 83	184	184	184	184	189	189	138	148	123	123		

Allele frequencies were calculated where the highest value belongs to the 184 bp long fragment at the locus PceGA025, following with 189 bp long products of BPPCT 002 (2. Figure).

2. Figure: Allele frequency of the 4 microsatellites which were used to analyze the 20 apricot samples



4.2 Statistical analysis of SSR results

Evaluation of the 4 markers is in 5. Table, where among others number of alleles, observed and expected heterozygosity and Fixation index were determined. The highest number of alleles belongs to AMPA-105 and BPPCT 002. However, the expected heterozygosity index is lower regarding BPPCT 002 and the highest is AMPA-105. According to the expected heterozygosity out of these primer pairs PceGA025 had the lowest values, but still satisfactory result.

Fixation index is a measure of population differentiation due to genetic structure, in this study AMPA-105 and BPPCT030 had the highest values.

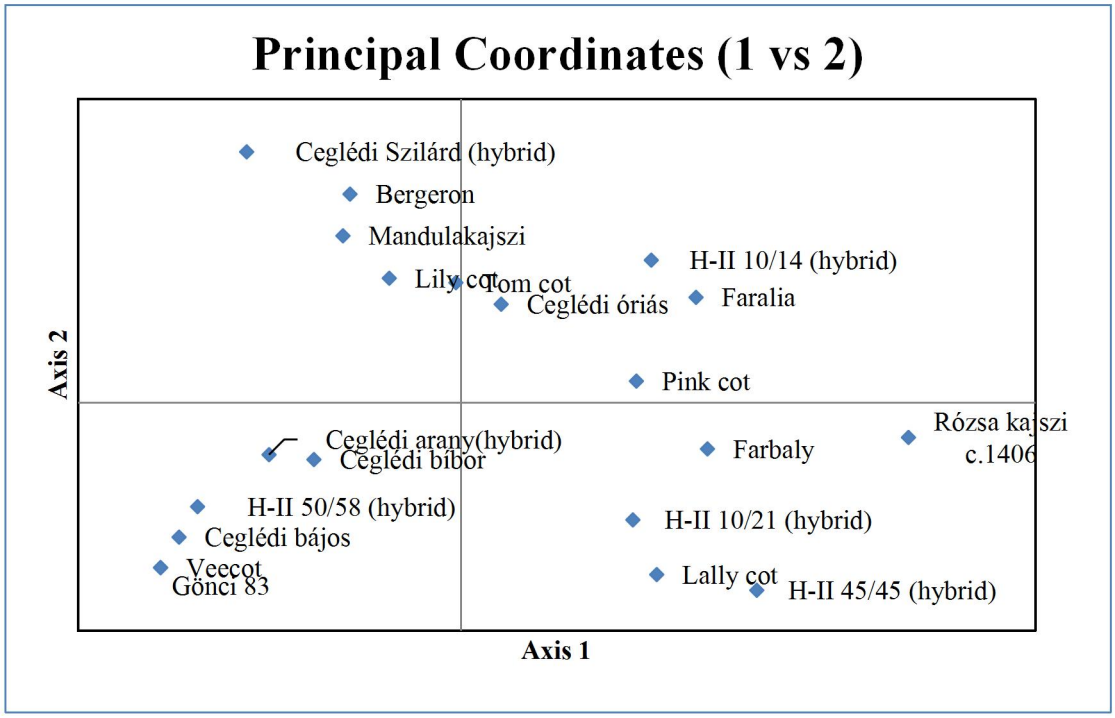
5. Table: Evaluation of the primer pairs efficiency in the case of 20 *Prunus armeniaca* L genotypes

Locus	**N**	**Na**	**Ne**	**I**	**Ho**	**He**	**uHe**	**F**
AMPA-105	20	7	5,714	1,820	0,55	0,825	0,846	0,333
PceGa25	20	5	2,402	1,145	0,5	0,584	0,599	0,143
BPPCT 002	20	7	2,996	1,356	0,55	0,666	0,683	0,174
BPPCT 030	20	4	2,749	1,107	0,5	0,636	0,653	0,214
Mean	20	5,75	3,466	1,357	0,525	0,678	0,695	0,216

Na = No. of Different Alleles; Ne = No. of Effective Alleles = 1 / (Sum pi^2); I = Shannon's Information Index = -1* Sum (pi * Ln (pi));Ho = Observed Heterozygosity = No. of Hets / N; He = Expected Heterozygosity = 1 - Sum pi^2; uHe = Unbiased Expected Heterozygosity = (2N / (2N-1)) * He; F = Fixation Index = (He - Ho) / He = 1 - (Ho / He); Where pi is the frequency of the ith allele for the population & Sum pi^2 is the sum of the squared population allele frequencies.

Principal coordinates were used to investigate genetic variation between genotypes (3. Figure). Veecot and Gönci 83 are the furthest to Rózsakajszi C.1406.

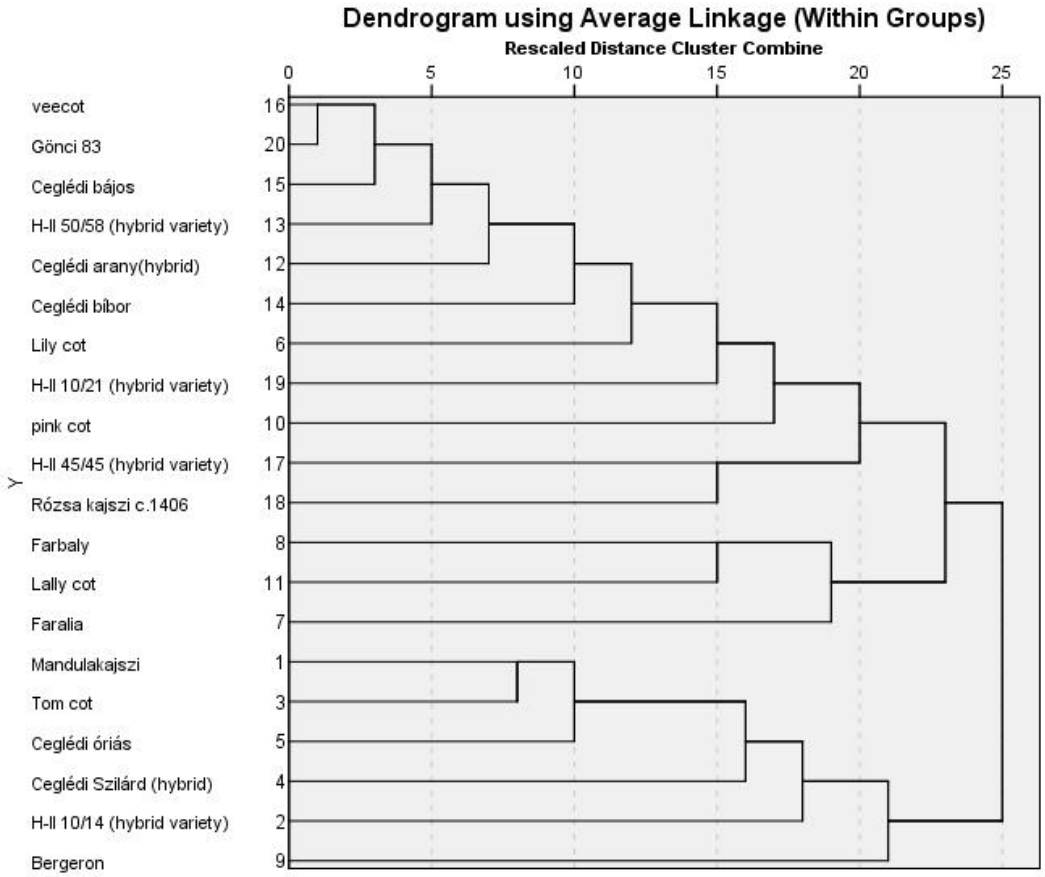
Our results can be group into 3 groups. One of the groups contains mostly tolerate, moderately tolerate genotypes and in the other group mostly the very tolerate genotypes can be found.



3. Figure: Principal coordinates of 20 apricot samples with 4 analyzed microsatellites

4.3. Hierarchical analysis of *Prunus armeniaca* L. genotypes

Dendrogram was drawn by using SPSS statistical program with 4 markers and 20 genotypes (4. Figure). According to my results only Gönci 83 and Veecot cannot be distinguished from each other every other case, these 4 markers were enough. Two major groups can be observed on the dendrogram. One of them contains genotypes which are tolerant, moderately tolerant to cold and chilling.



4. Figure: SSR based dendrogram with 20 *Prunus armeniaca* L. genotypes

5. Conclusions and recommendations

Out of the 4 SSRs analyzed in my study, AMPA105 was the most informative due to its high degree of polymorphism, which makes it more ideal marker for MAS breeding programs. PceGa25, on the other hand, was the least informative marker (showed the highest allele frequency) and thus exhibited the highest degree of similarity, it was deemed less ideal than AMPA105 for differentiating related Prunus genotypes for cold tolerance (resistance) and cold susceptibility (non tolerance).

The highest degree of genetic diversity observed amongst genotypes studied for cold tolerance or susceptibility provides a basis of artificial selection in plants through MAS breeding technique to develop better performing cultivars that are more tolerant to frost/ cold weather conditions by using the analyzed cold tolerant genotypes' germplasm. The findings from this study will also provide more insight for more scientific future researchers interested in the Prunus crop to carry out further analysis on more other genotypes to analyze their tolerance or susceptibility to cold or frost weather conditions. The results from this study will undoubtedly be crucial as a basis for selection for cold tolerant/ resistant genotypes from this study for use in the future MAS breeding programs.

6. Summary

Rosaceae, usually referred to as the rose family, is the same family as apricots. It is a diploid species with eight pairs of chromosomes (2n=16), and it is distinguished by a significant amount of heterozygosity. However, the absence of a high-quality reference genome for the apricot has turned into a handicap that has significantly hampered our understanding of how the genetic background, evolutionary diversity, and demographic diversity of the apricot influence accessions of phenotype. The juvenility phase of apricots lasts only approximately 2-3 years, which is comparably short compared to the majority of other fruit tree species, which require 6–10 years.

Twenty randomly selected samples of different apricot genotypes were used in the investigation. As soon as possible after leaf collection, DNA was extracted using the Nucleospin Plant II micro kit. The ND-1000 NanoDrop Spectrophotometer was used to measure the extracted DNA concentrations, and if necessary, DNA was diluted to 20 ng/µl. This followed the amplification of DNA fragments by the touch down PCR technique in which the multiple primers(forward and reverse) were used for each sample, the primers used included AMPA-105, BPPCT 002, BPPCT 007, BPPCT 030, MA039a, PceGA25, UDP 98 410, samples were labelled i to xx (one to twenty).

In the study, agarose gel electrophoresis was used to analyze the quality and quantity of the DNA concentration before analyzing the PCR results. Both experiments made use of 1% TBE agarose gels, which were made using a 5x TBE stock solution.

The sizes of the alleles were determined using the ALFexpress® II DNA Analyzer (Amersham Bioscience), which employs a polyacrylamide gel electrophoresis technology to separate DNA fragments utilizing automated detection of fluorescently tagged DNA molecules.

After samples were separated on a 6% polyacrylamide gel, the fragment lengths were detected using an ALF. Unfortunately, not all samples were examined due to the university's closure; only 4 (AMPA-105, BPPCT 002, BPPCT 030 and PceGA025) of the 7 markers tested have been reviewed by ALF.

From the study, 23 different allele sizes were successfully detected with aforementioned 4 markers.

The SSR primers generated allele sizes in the range of 176 to 208 for AMPA-105, 170 to 194 for PceGa25,181 to223 for BPPCT002 and 138 to 150 for BPPCT030.

All the four markers that used in this study were viable to distinguish the genotypes from each other except in the case of Veecot and Gönci 83. From the study, mean of the different alleles (Na) was 5,75, mean of effective alleles (Ne) was 3,466; mean of observed heterozygosity (Ho) was 0,525 while the mean of expected heterozygosity (He) was 0.678. Regarding heterozygosity index 0.5 and above value suggest an ideal applicable marker to genotype Prunus species. Thus, all the analyzed 4 microsatellites are suitable for distinguishing them.

PceGa25 with the highest allele frequency (monomorphic) while AMPA-105 showed the lowest allele frequency hence highest degree of polymorphism and so considered the most ideal marker for distinguishing the twenty cultivars studied. For the expected heterozygosity (He) the range was between 0.5 and 0.55 while the observed heterozygosity (Ho) ranged from 0.584 to 0.825. According to the results, the mean value of the He was generally greater than that of the Ho.

There was a significant amount of genetic variability within the genotypes under study, evidenced by the low similarity values across the 20 Prunus genotypes. As a result, the SSR are a perfect marker for differentiating the 20 genotypes under investigation since they are highly informative, codominant, multi-allele genetic markers that are transferable between related species and experimentally repeatable.

The twenty Prunus genotypes could be distinguished by these markers (SSR), which in this investigation AMPA-105 showed the highest degree of polymorphism. Therefore, SSR markers can be utilized an important method of choice for revealing genetic variation and identifying slightly different genotypes in breeding program ideally by the MAS technique. The research will be a helpful source of knowledge for developing molecular markers for cold tolerance as well as understanding the genetic variations in cold tolerance within the apricot species.

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9. Declaration

STUDENT DECLARATION

Signed below, Mugabi Julius, student of the Szent István Campus of the Hungarian University of Agriculture and Life Science, MSc Agricultural Biotechnology declare that the present Thesis is my own work and I have used the cited and quoted literature in accordance with the relevant legal and ethical rules. I understand that the one-page-summary of my thesis will be uploaded on the website of the Campus/Institute/Course and my Thesis will be available at the Host Department/Institute and in the repository of the University in accordance with the relevant legal and ethical rules. Confidential data are presented in the thesis: yes no*

Signature  Date; 2023/05/09

SUPERVISOR'S DECLARATION

As primary supervisor of the author of this thesis, I hereby declare that review of the thesis was done thoroughly; student was informed and guided on the method of citing literature sources in the dissertation, attention was drawn on the importance of using literature data in accordance with the relevant legal and ethical rules.

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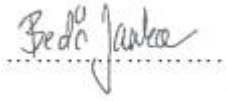
Date: __Gödöllő___ 2023_____ month _5__ day _9__  Signature