T.C.

ÇANAKKALE ONSEKIZ MART UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETIC

MOLECULAR EVALUATION OF COMMERCIAL HYBRID SUNFLOWER (*Helianthus annuus* L.) GERMPLASM FOR FERTILITY RESTORATION GENES

MASTER OF SCIENCE THESIS

GÖKTUĞ SERBEZLER

Thesis Supervisor

Assoc. Prof. Özge KARAKAŞ METİN

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ETHICAL STATEMENT

Çanakkale Onsekiz Mart Üniversitesi Lisansüstü Eğitim Enstitüsü Tez Yazım Kuralları'na uygun olarak hazırladığım bu tez çalışmasında; tez içinde sunduğum verileri, bilgileri ve dokümanları akademik ve etik kurallar çerçevesinde elde ettiğimi, tüm bilgi, belge, değerlendirme ve sonuçları bilimsel etik ve ahlak kurallarına uygun olarak sunduğumu, tez çalışmasında yararlandığım eserlerin tümüne uygun atıfta bulunarak kaynak gösterdiğimi, kullanılan verilerde herhangi bir değişiklik yapmadığımı, bu tezde sunduğum çalışmanın özgün olduğunu, bildirir, aksi bir durumda aleyhime doğabilecek tüm hak kayıplarını kabullendiğimi taahhüt ve beyan ederim.

In this thesis study that I prepared following the Thesis Writing Rules of School of Graduate Studies of Çanakkale Onsekiz Mart University; I declare that I have obtained the data, information, and documents I presented in the thesis within the framework of academic and ethical rules, I have presented all information, documents, evaluations, and results following scientific ethics and ethical rules, I cited all the works that I used in my thesis study by making appropriate reference, I did not make any changes in the data used and that the study I presented in this thesis is original. Otherwise, I undertake and declare that I accept all loss of rights that may arise against me.

Göktuğ SERBEZLER
23/08/2022

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ÖZET

TİCARİ HİBRİT AYÇİÇEĞİ (*Helianthus annuus* L.) GERMPLAZMININ FERTİLİTE RESTORASYON GENLERİ İÇİN MOLEKÜLER DEĞERLENDİRİLMESİ

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Danışman: Doç. Dr. Özge KARAKAŞ METİN
23/08/2022, 60

Süs bitkisi ve çerezlik olarak da yaygın olarak kullanılan ayçiçeği (*Helianthus annuus* L.), FAO'ya göre küresel çapta en çok tüketilen dördüncü bitkisel yağ kaynağıdır. Dünya genelinde ticari olarak kullanılan ayçiçeği türleri verimlerini yükseltmek, hastalıklara ve stres koşullarına dayanıklı hibrit türler üretmek amacıyla farklı özelliklerde ki yabani ayçiçeği türleri ile çaprazlanmaktadır. Ticari amaçlı üretilecek ayçiçeği hibrit tohumlarının üretimi sırasında diğer bitki türlerinde olduğu gibi kendine tozlaşma büyük bir engel oluşturmaktadır. Hibrit tohum üretimin de kendine tozlaşmayı önlemek için genel olarak bitkilerde, fonksiyonel polen üretimini engellediği için sitoplazmik temelli erkek kısırlığı (CMS) özelliği aranır. Sitoplazmik temelli erkek kısırlığına dayalı ticari hibrit tohum üretim çalışmalarında doğurganlık restorasyon genleri (*Rf*), polen verimliliğinin düzenlenmesinde rol aldığı için temel öneme sahiptir. Bu durum doğurganlık restorasyon genlerini, sitoplazmik erkek kısırlığı kadar önemli kılmaktadır. İstenilen gen bölgesiyle yakından bağlantılı moleküler markörlerin kullanımına dayanan markör destekli seçim (MDS) yöntemi geleneksel ıslah yöntemi yerine son yıllarda yaygın olarak kullanılmaya başlanmıştır. Fakat henüz ülkemizdeki ticari ayçiçeği genotipleri için doğurganlık restorasyon gen bölgesiyle yakından bağlantılı moleküler markör taramaları yapılmadığı için hibrit tohum üretme çalışmalarında geleneksel ıslah yöntemi kullanılmaktadır. Fakat bu yöntem çok sayıda çaprazlama yapılmasını gerektirmektedir. Bizde çalışmada, şimdiye kadar dünya genelinde ayçiçeğinde tespiti yapılan basit dizi tekrarı (SSR) markörlerini kullanarak, ülkemizde ticari olarak kullanılmakta olan ayçiçeği genotiplerinde tarama

çalışması gerçekleştirdik. Çalışmada Trakya Tarımsal Araştırma Enstitüsü'nden elde edilen erkek steril ve restorasyon genotipli ve bu genotipteki bireyler kullanılarak elde edilen F_2 bireylerinin tarama çalışmaları yapılmıştır. Şimdiye kadar tespiti yapılan 7 restorasyon gen bölgesine ait 21 SSR markörü, restorasyon bölgesine yakın olmaları ve aynı lokuslarda bulunmaları sebebiyle seçilmiştir. Çalışma sonucunda elde edilen veriler ülkemizde yapılacak ıslah çalışmalarında kullanılabilme potansiyeli taşımaktadır.

Anahtar Kelimeler: Fertilite Restorasyon Genleri, *Helianthus annuus* L., Sitoplazmik Erkek Kısırlığı, Markör Destekli Seçim, BSA, SSR

ABSTRACT

MOLECULAR EVALUATION OF COMMERCIAL HYBRID SUNFLOWER (*Helianthus annuus* L.) GERMPLASM FOR FERTILITY RESTORATION GENES

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Master of Science Thesis in Molecular Biology and Genetic

Advisor: Assoc. Prof. Özge KARAKAŞ METİN

08/23/2022, 60

According to FAO, sunflower (*Helianthus annuus* L.), also widely used as an ornamental plant and confectionery, is the fourth most consumed vegetable oil source globally. Sunflower species used commercially worldwide are crossed with wild sunflower species with different characteristics to increase their yield and obtain hybrid species resistant to diseases and stress conditions. Self-pollination is a major obstacle during the production of sunflower hybrid seeds to be produced for commercial purposes. In order to prevent self-pollination in hybrid seed production, the cytoplasmic-based male sterility (CMS) feature is sought in plants as it inhibits functional pollen production. Fertility restoration genes (*Rf*) are of fundamental importance in commercial hybrid seed production because they are involved in the regulation of pollen productivity. The traditional breeding method is used in hybrid seed production studies since molecular marker screenings closely related to the fertility restoration gene region have not yet been performed for commercial sunflower genotypes in our country. However, this method requires a large number of crossovers. In our study, we carried out a screening study on sunflower genotypes that are being used commercially in our country, using simple sequence repeat (SSR) markers that have been made in sunflowers all over the world so far. In the study, screening studies of F_2 individuals obtained from Thrace Agricultural Research Institute with male sterile and restoration genotype and individuals with this genotype were carried out. 21 SSR markers belonging to 7 restoration gene regions detected so far were selected because they are close

to the restoration region and are located at the same loci. The data obtained as a result of the study has the potential to be used in breeding studies to be carried out in our country.

Keywords: Fertility Restoration, *Helianthus annuus* L., Cytoplasmic Male Sterility, Marker Assisted Selection, BSA, SSR

CONTENTS

Page No

SYMBOLS AND ABBREVIATIONS

%	Percent
μg	Microgram
μl	Microliter
°C	Degrees Celsius
AFLP	Amplified Fragment Length Polymorphism
bp	Base pair
cM	Centi Morgan
CMS	Cytoplasmic Male Sterility
DArT	Diversity Array Technology
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylene Diamine Tetra Acetic Acid
FAO	Food and Agriculture Organization of the United Nations
g, G	Gram
Ha	Harvested area
ISSR	Inter Simple Sequence Repeat
Kb	Kilo base
Kg	Kilogram
L	Liter
M	Molar
MAS	Marker Assisted Selection
Mg	Miligram
Min	Minute
MS	Male Sterility
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
nm	Nanometer
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
Rf	Fertility Restorer
RFLP	Restriction Fragment Length Polymorphism

RNA	Ribonucleic acid
Sec	Second
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeat
Ta	Annealing temperature
TAE	Tris-acetate-EDTA
U	Unit

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1
INTRODUCTION

1.1. Introduction to Sunflower

Sunflower (*Helianthus annuus* L.) is one of the most important oil crops in the world. It is an annual species and a member of the *Helianthus* genus belonging to the Compositae (Asteraceae) family; its chromosome number is 17. The words “helios” meaning sun, and “anthus”, meaning flower, are camed together to produce Helianthus, the name of the genus.

Kingdom	Plantae
Section	Magnoliophyta
Class	Magnoliopsida
Order	Asterales
Family	Asteraceae
Subfamily	Asteroideae
Genus	*Helianthus*
Species	*Helianthus annuus* L.

Figure 1. Scientific classification of cultivated sunflower

The genus *Helianthus* also includes diploid, tetraploid, and hexaploid species. The genus *Helianthus* consists of 51 species with 37 perennial and 14 annual species (Schilling et al., 1981). *H. annuus* L., the most common sunflower, and *H. tuberosus* species are two

important species cultivated for food purposes. Other cultivated species are often used as ornamental plants (Fernandez et al., 2010).

The most common cultivated sunflower (*H. annuus* L.) consists of 3 main groups.

These are:

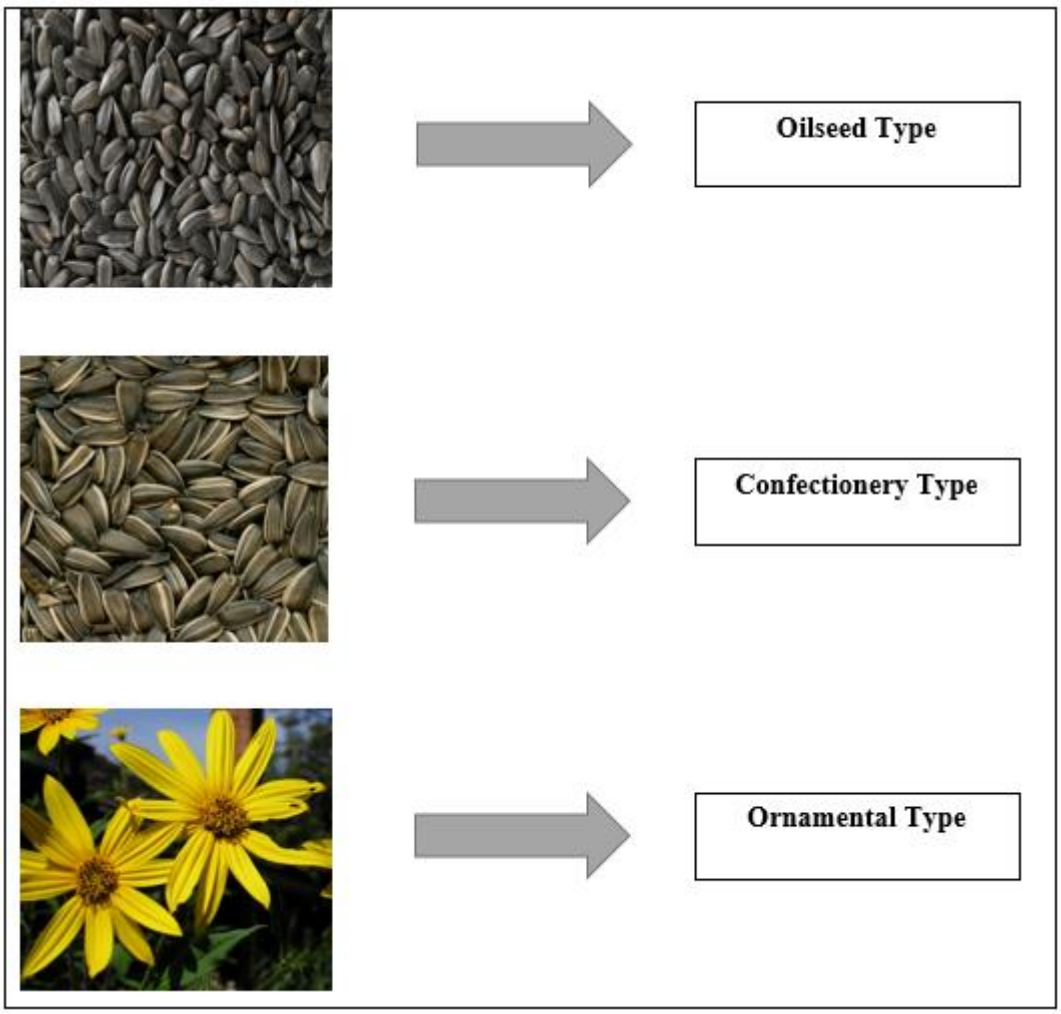


Figure 2. Production purposes of commercial sunflower

However, most of the sunflower production consists of oilseed species (Miller et al., 1997).

1.2. The History of the Sunflower

Based on archaeological evidence, the sunflower, a plant of North American origin, is thought to have been domesticated by Native Americans as early as 2300 BC. For this reason, it is believed that sunflower domestication preceded beans and maize. Throughout history, every part of the sunflower has been used for different purposes in daily life; its oil was used in cooking, its bark and petals were used for dye preparation, and its seeds were used as flour and even as coffee. After the sunflower was brought to Europe in the 1500s, it began to be used for medical purposes such as treating snake bites and improving eyesight (Heiser et al., 1969). In the middle of the twentieth century, sunflower became a significant plant species worldwide because of the excellent breeding efforts in Russia. After the cytoplasmic male sterility (CMS) discovered by Leclercq in 1969 and fertility restorer (*Rf*) genes discovered by Kinman in 1970, sunflower has been commercially produced in many parts of the world.

1.3. Importance of Sunflower

85% of the total edible oil production in the world is obtained from four vegetable sources, one of them being sunflower (FAO, 2018). Sunflower is a significant source of vegetable oil worldwide due to its high oil content (40-52%), cholesterol-free, and unsaturated fatty acids ranging from 85-91%. In addition, sunflower oil is rich in vitamin content; these are vitamins A, D, E, and K. The by-products of the seed (seed cake) are also used because it is a rich source of protein (35%) and carbohydrates (18-20%) for animals (Hossam et al., 2012).

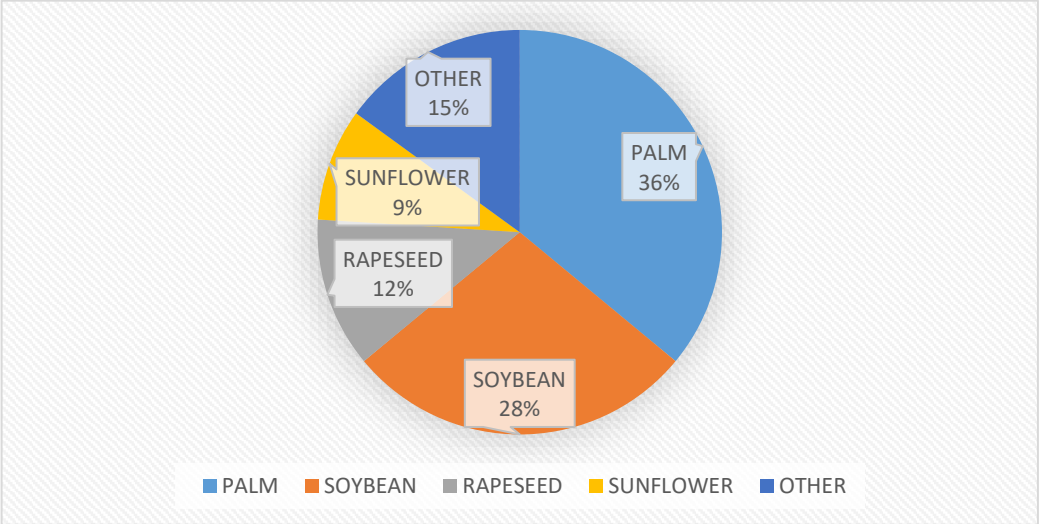


Figure 3. The world's production of edible oil (FAO, 2018).

According to FAO's data, sunflower ranks fourth in vegetable oil production in the world with 9%, while it ranks first in vegetable oil production in our country with 56.2%. Although many types of oil seeds are cultivated in our country, such as cottonseed, soybean, peanut, poppy, sesame, rapeseed, and safflower, sunflower stands out as the oil plant with the highest cultivation area and production. Especially in the Marmara and Thrace Regions, it is the main plant in crop rotation. Wide adaptability, being able to be grown in dry and irrigated conditions, and being very suitable for mechanization make the sunflower the most crucial oilseed plant for our country (Kaya et al., 2003).

Figure 4. Türkiye sunflower production areas (Republic of Türkiye Ministry of Agriculture and Forestry, 2019)

1.4. Why does Türkiye import sunflower oil?

In Türkiye, a total of 2.10 million tons of production was realized in 2019/2020 on an area of 752 thousand hectares with an average yield of 279 kg/da. However, Türkiye's sunflower production is not sufficient even for domestic consumption. Since there is a constant increase in vegetable oil consumption due to rapid population growth and per capita consumption in Türkiye, oil production cannot meet the consumption. Therefore sunflower is imported to meet the growing vegetable oil shortage. For this reason, increasing production areas and breeding studies in sunflowers are necessary (Konyalı, 2018; Anonymous, 2021).

1.5. Breeding Studies of Sunflower

Hybrid studies in sunflower aim to develop individuals resistant to biotic and abiotic stress, increase yield, and meet unique demands. These are generally as follows;

a) developing high seed and oil yield hybrids that are resistant to herbicides, dominant diseases, and drought,
b) modifying achene and kernel properties as well as protein and oil content of confectionery sunflower
c) producing specific fatty acid and tocopherol compositions in the food and non-food industry
d) changing plant height, flower color, and flowering time of ornamental sunflowers (Jocic et al., 2015; Dimitrijevic et al., 2018).

In sunflower production, using open-pollinated varieties was the only option until the early 1970s (Vear, 2016).

The discovery of the cytoplasmic male sterility (CMS) source and the corresponding fertility restorer genes opened the way for hybrid production in sunflowers discoveries enabled the use of heterosis for hybrid development, resulting in higher seed and oil yield in sunflowers. Thus, the sunflower has become an important crop worldwide (Bohra et al., 2016)

1.6. CMS/Rf System

Cytoplasmic male sterility (CMS) and fertility restoration genes (*Rf*) are two crucial parts of a system used in hybrid seed production. They are widely used in hybrid seed production of cultivated plants such as sunflower, maize, rice, and others. Male sterility is a maternally inherited condition; the plant is unable to produce functional pollen (Laser et al., 1972). Male sterility has generally been shown to result from mitochondrial defects. Figure 5 shows us the CMS-related regions in the mitochondrial genomes of species (Schnable et al., 1998).

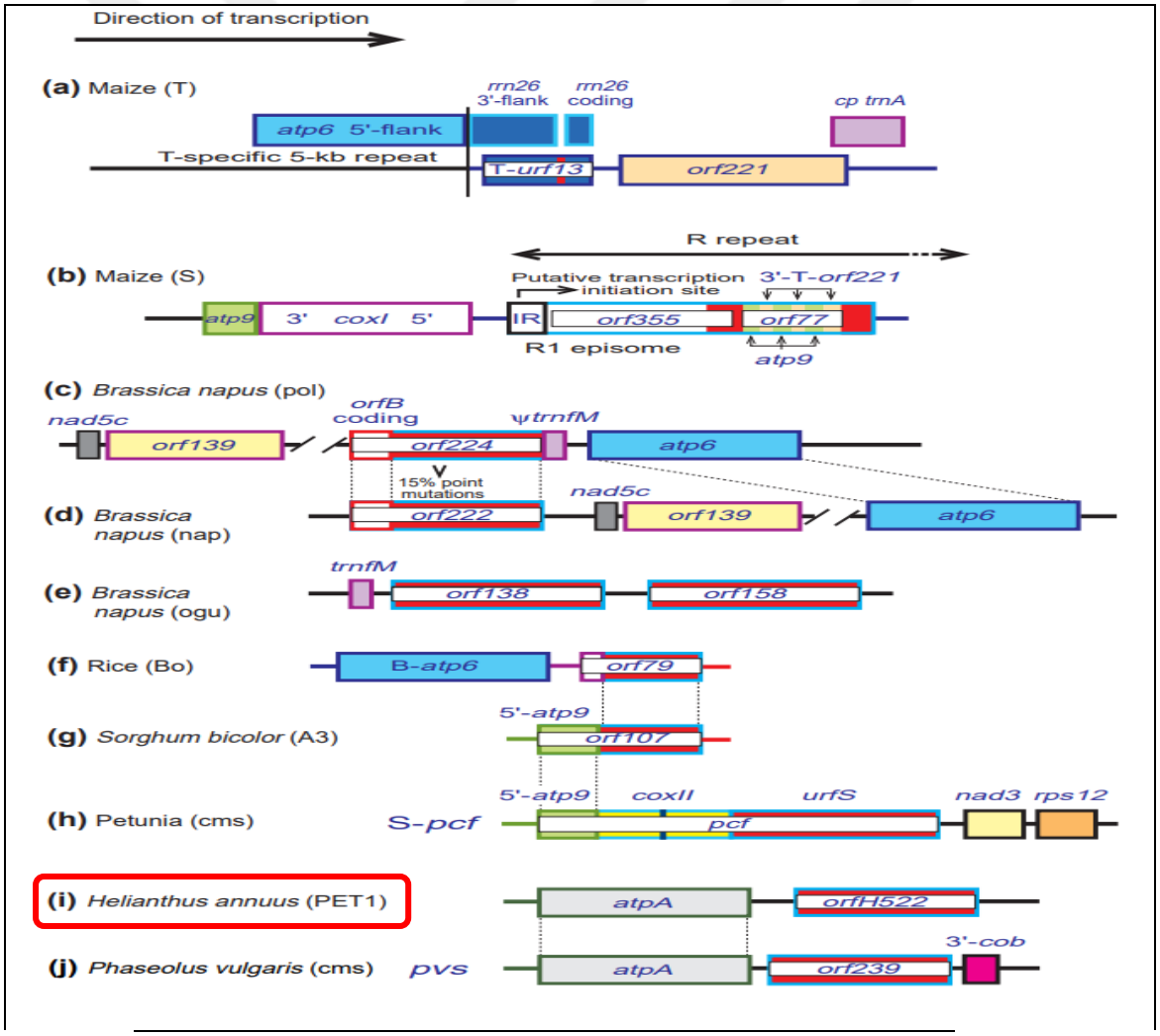


Figure 5. CMS-related regions in the mitochondrial genomes of species (Schnable et al., 1998)

Nuclear genes, called fertility restorers (*Fr*) or restorer fertility (*Rf*), were discovered by Kinman in 1970. They have the ability to suppress the male-sterile phenotype and thus restore pollen production to plants carrying the deleterious mitochondrial genome. CMS-Rf systems make easy hybrid seed production by eliminating the need for laborious hand emasculation and preventing self-pollination. The *Rf* allele from the pollen parent repair fertility and seed production in the heterotic hybrid progeny. In addition to commercial exploitation of the CMS-Rf system, it offers an opportunity to investigate the regulation of mitochondrial gene expression by a nuclear gene in multicellular organisms.

Since the first CMS was reported in sunflower in 1969, 72 CMS sources have been identified (Serieys 2005), but only the first CMS PET1 (1969) has been used to produce almost all commercial sunflower hybrids. Inheritance studies showed that nearly all restorer lines for CMS PET1 in sunflower breeding carry the same restorer gene, *Rf1* (Serieys, 1996).

The CMS system is a common phenomenon found in more than 150 plant species (Laser et al., 1972). While CMS line can be easily produced, finding restorer genes are much more difficult, especially when using the cultivated sunflower, because fertility restorer genes are isolated from the wild species (Vranceanu et al., 1978). Although many sources of CMS have been discovered, only seven *Rf* genes have been identified.

Many mapping studies have been done for *Rf* genes. The *Rf1* gene was mapped to linkage group (LG) 6 in the RFLP map by Gentzbittel et al. in 1995 and to LG 2 by Jan et al. in 1998. Also, the *Rf1* gene, like the *Rf5* gene, has been mapped in the SSR map by Qi et al. in LG 13. The Msc1 gene was mapped in LG 12 in 1999 in the RFLP map by Gentzbittel et al. In the SSR map, the *Rf3-RHA 340* and *Rf3-RHA 280* genes were mapped to LG7 by Liu et al. in 2012, and the *Rf4* gene to LG 3 by Feng et al. in 2008. And the *Rf-PEF1* gene was also mapped in the AFLP map by Schnabel et al. 2008 in LG 13.

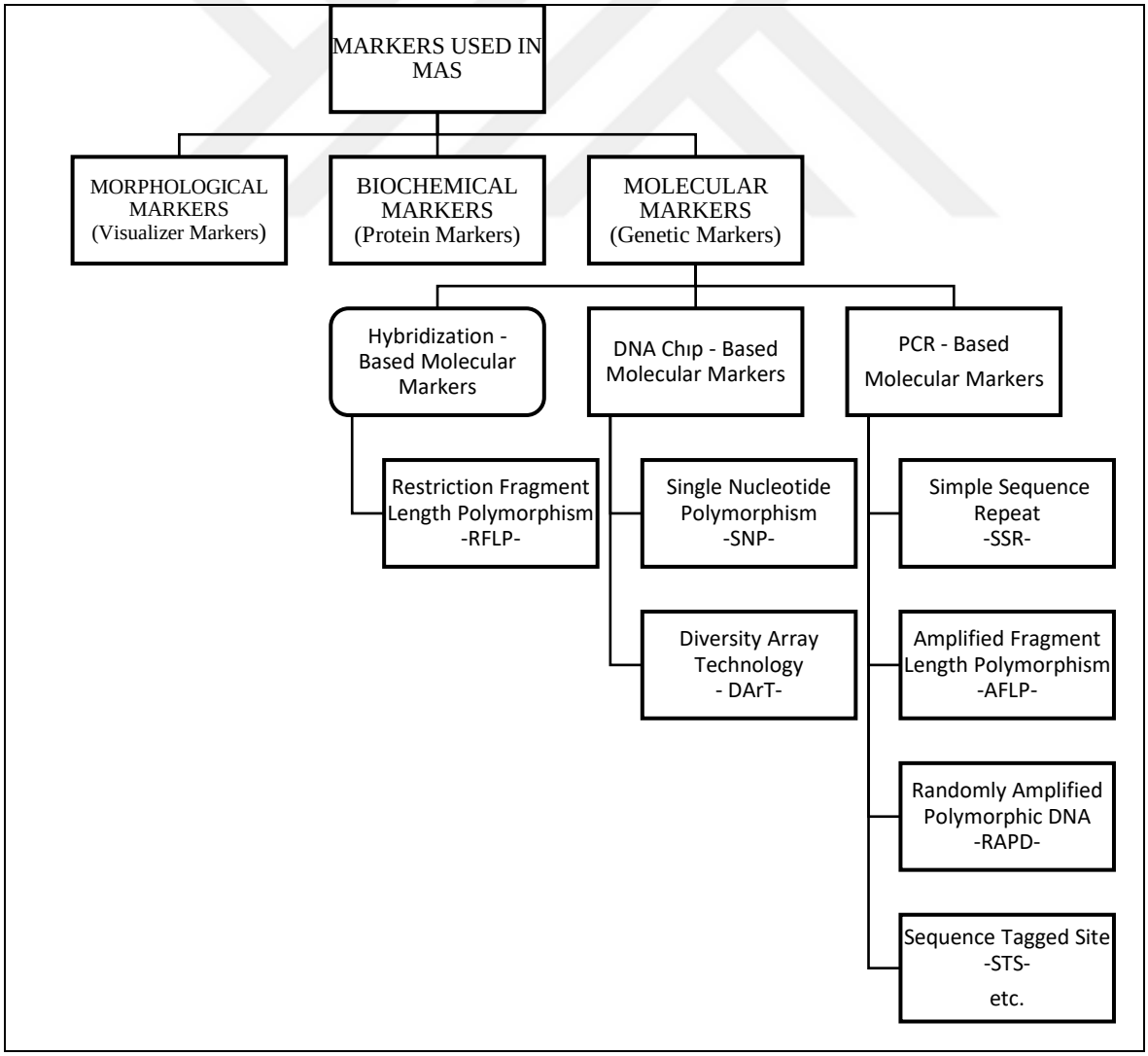
Alternative CMS/Rf gene systems could expand the diversity of the sunflower crop and reduce the risks inherent with using a single CMS/Rf system, such as cytoplasmic uniformity and increase the genetic diversity of sunflower hybrids to everchanging environmental stress and disease. Also, the identification and characterization of additional CMS/Rf gene systems will enrich the knowledge of the interactions between the cytoplasm and nuclear genes (Liu et al., 2013).

1.7. Marker Assisted Selection

Marker-assisted selection (MAS) is an indirect selection process in which traits such as productivity, disease resistance, abiotic stress tolerance, and quality are selected based on a marker linked to a trait of interest rather than the trait itself. The development of marker-assisted selection techniques instead of breeding techniques that require a long time and are labor intensive has accelerated the breeding studies and facilitated obtaining special varieties. Markers used in MAS are divided into three groups morphological, biochemical, or molecular markers.

Table 1

Summary of markers that are used in MAS.



1.7.1. Morphological Markers

Morphological markers depend on the phenotypic selection of the species traits, which is controlled by a single locus and can be changed by environmental conditions. It refers to visible selective traits that distinguish a plant or a group from others within a plant population. That's why they are also called visualizer markers. Features such as fruit skin, leaf shape, and flower color form the markers of this group.

Morphological markers can be easily identified because they are not so many, and it is easy to distinguish a particular dominant allele from a recessive one. However, there are some critical difficulties in using morphological markers. These are, they do not allow the determination of heterozygous genotypes, and the environment can change the phenotypic traits used as morphological markers.

1.7.2. Biochemical Markers

Biochemical markers are protein-based markers developed to eliminate the problems caused by environmental conditions affecting morphological markers. That's why they are also called protein markers.

Protein markers are divided into enzyme proteins (isoenzyme) and non-enzyme proteins (storage proteins such as gliadin and glutenin).

They are marker types with fast, reliable, and repeatable results and are not affected by environmental conditions. And protein markers are cheap because isoenzymes encoded by different alleles of a gene or encoded by different genes are not expensive to study. However, these isoenzymes may belong to several loci or different tissue types and may have different developmental stages.

1.8. Molecular Markers

DNA sequences that express any gene region or DNA fragment associated with a gene region in the organism’s genome, have no biological effects, and are passed on from generation to generation are called molecular markers (Semagn et al., 2006). Molecular markers are also known as DNA markers because they are detected by analysis at the DNA

level, unlike morphological markers based on visible features and biochemical markers based on proteins that are the product of genes. In general, DNA-based marker systems are more effective than other marker systems. Molecular markers can be used in the whole plant, at the tissue and cellular level. DNA markers are stable, can occur in all tissues, and are not affected by environmental conditions (Fernie et al., 2006).

Molecular markers are divided into three groups as hybridization-based makers, polymerase chain reaction-based markers, and DNA chip-based markers.

1.8.1. Hybridization-Based Molecular Markers

This method is based on creating hybrid molecules between DNA/DNA or DNA/RNA. It is used to determine the locations of the desired DNA fragments in the genome and investigate the nucleotide sequences of these fragments.

RFLP (Restriction Fragment Length Polymorphism), the first molecular marker system developed by Soller and Bechman in 1983, is based on the hybridization of DNA fragments (probe) cut with restriction enzymes to DNA of similar or identical sequence in the DNA sample under investigation. Utilizing RFLP markers requires the use of short-lived radioactive material, a limiting factor. Other limiting factors; are obtaining high concentration and pure DNA, and restriction cutting requires long and expensive isotopic labeling. It has become imperative to search for new marker systems to overcome these difficulties.

1.8.2. DNA Chip-Based Molecular Markers

DNA microarray is a microscopic DNA spot formed in an array by attaching it to a solid surface such as a glass, plastic, or silicon chip to simultaneously monitor the expression level of thousands of genes. There can be thousands of these spots in a microarray. In the manufacture of DNA chips, DNA probes similar to synthetic probes known as allele-specific oligonucleotides (Allele-Specific Oligonucleotides, ASO) are used in semiconductor industry technology.

DArt (Diversity Array Technology) is a molecular marker technique based on microarray hybridization that is a high throughput genome analysis, which is the application of DNA chip technology to DNA polymorphism technology. This technology allows simultaneous typing of several hundred polymorphic loci spanning the genome (Wenzel et al., 2004). It measures the presence or amount of a unique DNA segment originating from a population or genomic DNA of an organism.

1.8.3. PCR-Based Molecular Markers

Developed by Mullis in 1983, this technique has allowed the development of many PCR-based methods. PCR is based on amplifying the target genetic material using a pair of oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), a buffer system, and a thermostable DNA polymerase enzyme.

PCR technique; It is used in DNA sequence analysis and DNA mapping, DNA fingerprinting studies, genetic mapping, human genome project research, forensics, calculation of polymorphism between species, evolution, determination of seed purity in plants, cloning, identification of genetically modified organisms, mutagenesis studies and comparison of gene expressions.

Some of the commonly used marker systems based on PCR; are SSR (Simple Sequence Repeat, microsatellite), ISSR (Inter Simple Sequence Repeat), Expressed sequence tag-derived simple sequence repeat markers (EST-SSRs), AFLP (Amplified Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), and STS (Sequence Tagged Site).

1.9. Chosen Ideal Marker Method

An ideal polymorphic marker should be highly polymorphic, co-dominant inherited, frequently found in the genome, neutral to environmental conditions, high reproducible capacity, and easy to access and assay (Arif et al., 2010). Comparisons of different molecular markers via working principles and positive and negative aspects are given in Table 2.

In this study, we chose to use the SSR marker method because we did an intra-species analysis; the method offers high polymorphism and is reproducible, cost-effective, and reliable. Also, the SSR sequences are predetermined for sunflowers (*Helianthus annuus* L.).

Table 2

Comparison of different molecular markers.

Marker Type	Working Principle	Positive Aspects	Negative Aspects
SSR	PCR of simple sequences	-Widespread in the genome -High repeatability -Pretty good genome coverage -High polymorphism -Easy to automate -Having multiple alleles	-Inability to cross-species -Requires sequence knowledge
RAPD	Amplification with random primers	-Widespread in the genome -Being suitable for automation -Requires a small amount of DNA	-Lack of probe and primer information -Being dominant -Not repeatable
RFLP	Restriction and Southern blot hybridization	-Widespread in the genome -Being codominant -High repeatability -Cross-species availability -No need for sequence information	-Requires a high amount of quality DNA -The need for radioactive labeling -Requires cloning and probe characterization
AFLP	Restriction and selective PCR amplification	-Widespread in the genome -High polymorphism -Does not need sequence information -Cross-species use	-Being very sensitive to changes in the materials used -Does not create stable maps (no repeatability)
EST-SSR	PCR amplification of SSR from expressive regions (exons)	-Cross-species use -High-quality band profile -Good genome coverage -Not radioactively labeled -Easy to automate	-Low polymorphism
STS	PCR amplification of short sequences	-Pretty good genome coverage -High repeatability -Not radioactively labeled -Multiple uses of filters	-Requires sequence knowledge -Requires cloning and probe characterization

1.10. SSR (Simple Sequence Repeat, Microsatellite)

Molecular markers are crucial for understanding genome organization and provide significant advantages in developing new lines and identifying differentiation between the initial germplasm (Santalla M. et al., 1998). The development of molecular markers in sunflower is high level, and over the years, different types of markers have been developed for marker-assisted selection (MAS). Numerous other molecular markers are available to be used in sunflower breeding. (Wieckhorst S., 2012). RFLP (Restriction Fragment Length Polymorphism) (Berry S.T. et al., 1995), RAPD (Random Amplified Polymorphic DNA) (Lu Y.H. et al., 2000), AFLP (Amplified Fragment Length Polymorphism) (Quagliaro G. et al., 2001), and SSR (Simple Sequence Repeat) (Tang S. et al., 2002) markers are some of them.

SSR marker techniques, also called microsatellites, were chosen for this study. Because of the hexaploid structure of the sunflower genome, the microsatellite markers are cited as more effective tools for assessing their complex structure with the highly polymorphic informative structures of these markers when compared to other molecular markers systems. SSRs are short, simple sequence repeats that are mono- to hexa- nucleotide in length and occur throughout the genome due to errors in DNA replication (Moxon et al., 1999). Errors that may occur in replication are shown in Figure 6.

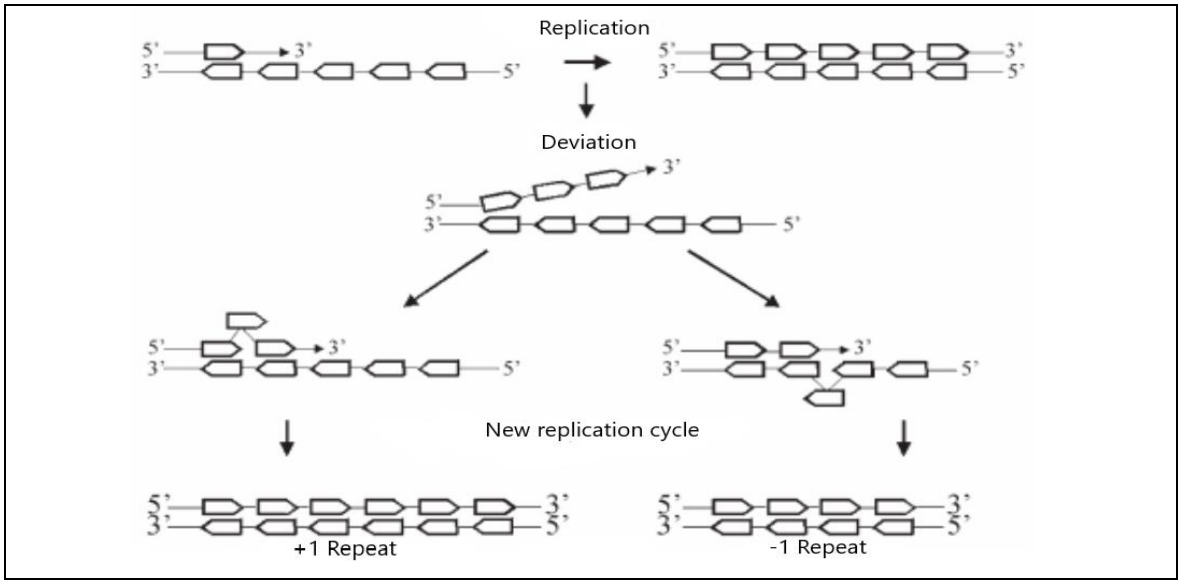


Figure 6. The error occurs during DNA replication

The difference in the number of consecutive SSR repeats results in the formation of DNA fragments of different lengths. Different alleles at a locus can be identified using these DNA sequences. In Figure 7, the separation of alleles using SSR is illustrated.

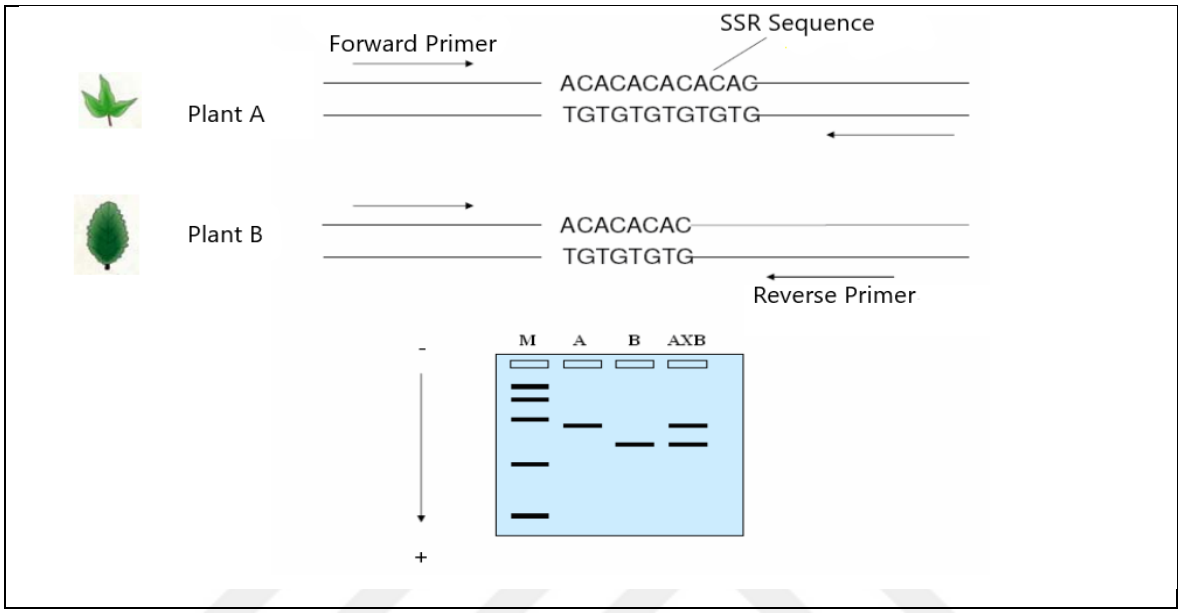


Figure 7. Schematic representation of the SSR technique

SSR repeats occur from mono- to hexa- nucleotide repeats, and (CA)n, (AAT)n, and (GATA)n are the most common SSR repeats in plants and animals as well (Jarne et al., 1996). Variation occurs by the change in the number of repeats.

Since SSR, which gives a lot of information in plants, is a reliable, high polymorphism rate, codominant, and highly reproducible marker method; it has been widely used in mapping, identification of cultivars, germplasm maintenance, identification of hybrids, analysis of gene pool variation and as markers for economically important traits in recent years (Gupta et al., 2000; Nimmakayala et al., 2009).

SSR markers were chosen for this study. Because of the hexaploid structure of the sunflower genome, the microsatellite markers are cited as more effective tools for assessing their complex structure with the highly polymorphic informative structures of these markers when compared to other molecular markers systems.

In addition to its high functionality the need for specific primers for each gene detection and the expensive and time-consuming nature of identifying and developing microsatellites from the first stage constitute the problematic part of the SSR method (Jones et al., 1997).

1.11. Bulk Segregant Analysis (BSA)

BSA (Bulk Segregant Analysis), developed by Michelmore in 1991, is based on finding polymorphic markers between two different DNA mixtures (bulk) belonging to individuals of a genetically unfolding population (plants of the F_2 or F_3 generation). These DNA mixtures are created from the DNA of individuals in the population that are different in terms of certain features or DNA regions, such as disease resistance and susceptibility. Still, they have a random structure for other genomic DNA regions. BSA was used to identify an RAPD marker linking resistance to *Liriomyza trifolii* which is a tomato pest moth. Similarly, Altınkut and Gözükırmızı 2003 obtained a microsatellite marker for water stress resistance in wheat using the BSA method.

Bulk segregation analysis can not discover new types of variation. However, it allows rapid scanning of many loci and identification of drop-down markers in the target region.

CHAPTER 2

PREVIOUS STUDIES

2.1. Detection of *Rf* Genes

Cytoplasmic male sterility (CMS) is a maternally inherited mutant trait, determined by often chimeric genes localized in the mitochondrial genome. Nuclear fertility restorer genes *Rf* (fertility restorers), whose products interact with the abnormal mitochondrial genome products, are required to suppress the CMS phenotype (Ivanov et al., 2007).

The CMS phenotype can occur from induced mutation or spontaneously (Kaul, 1988). The CMS system is a common phenomenon found in more than 150 plant species (Laser et al., 1972). While CMS lines can be easily produced, finding restorer genes is much more difficult, especially when using the cultivated sunflower. Because fertility restorer genes have been isolated from the same wild species (Vranceanu et al., 1978). Therefore, although there are many sources of CMS, only seven *Rf* genes have been identified.

Restoration of pollen productivity through dominant *Rf* genes is crucial in producing hybrid seeds based on CMS (Anisimova, 2009). But the biggest problem there is the detection of restorer genes. Identifying restorer genes or developing new restorer lines with the conventional breeding method is costly and time-consuming because it requires many crosses. Thus, identifying molecular markers closely linked to fertility-restoring genes reduces the time requirement, facilitating the breeding of new restorative strains (Isaacs et al., 2003). For this reason, mapping studies have been carried out within the *Rf* genes mapped so far. These are as follows.

Yue et al. mapped the *Rf1* gene in LG 13 using SSR and TRAP markers in 2010. Sajer et al. mapped the *Rf-PET-2* gene in LG 13 using SSR, STS and AFLP markers in 2020. Liu et al. mapped the *Rf3* gene in LG 7 using SSR and STS markers in 2012. Feng et al. mapped the *Rf4* gene in LG 3 using SSR and STS markers in 2008. Qi et al. mapped the *Rf5* gene in LG 13 using SSR markers in 2012. Liu et al. mapped the *Rf6* gene in LG 3 using SSR and EST-SSR markers in 2013. Talukder et al. mapped the *Rf7* gene in LG 13 using SNP markers in 2019.

CHAPTER 3
MATERIAL AND METHOD

3.1. Methods

3.1.1. Plant Materials

Four parental commercial sunflowers (*Helianthus annuus* L.) genotypes were used in this study. Two hybrid sunflower genotypes formed by crossing parental genotypes were obtained from the Trakya Agricultural Research Institute, Edirne. Name of genotypes, characteristics, and the number of individuals of each genotype are shown in Table 3.

Table 3

Commercial sunflower (*Helianthus annuus* L.) genotypes were used in this study.

Name of Genotypes	Characteristics	Number of Individuals
9728-A	Sterile parental	30
CL-217-R	Non-sterile parental	30
IMI-044-A	Sterile parental	30
3814-R	Non-sterile parental	30
9728-A x CL-217-R	Hybrid	100
IMI-044-A x 3814-R	Hybrid	100

Sterile: Source of CMS **Non-sterile**: Source of *Rf* gene

The plant's young leaves, suitable for DNA isolation, were preferred during the collection of the material. After the cut leaves were cleaned with alcohol, they were wrapped in aluminum foil and frozen with liquid nitrogen (-196°C). It was stored in liquid nitrogen and transported to the deep freezer (-80°C).

3.1.2. Genomic DNA Isolation

Genomic DNA isolation was performed according to Doyle and Doyle's (1987) CTAB-based genomic DNA isolation protocol for commercial sunflower plants. After about 80 mg of leaf tissue belonging to *9728-A, CL-217-R, IMI-044-A, 3814-R, 9728-A x CL-217-R, IMI-044-A x 3814-R* genotypes was frozen in liquid nitrogen, ceramic balls were added, and physical disintegration was provided in 1.5 ml Eppendorf tubes in the homogenizer device. 600 µl CTAB (60°C) solution and 8 mg of PVP (Polyvinylpyrrolidone) were added to each sample and incubated for 30 minutes at 60°C in a water bath inverting every 6 minutes. Afterward, 1.2 µl β-mercaptoethanol (β-ME) (0.2% of CTAB solution) was added to each sample and incubated again in a water bath at 60°C for 10 minutes. At the end of the time, it was left to cool at room temperature for 5 minutes. 600 µl of 24:1 chloroform: octanol was added to each sample, mixed by inverting 20-25 times, and centrifuged at 13,000 rpm for 15 minutes. Approximately 400 µl of supernatant in the tube was transferred to another tube, and 200 µl (50% of supernatant) of 5 M NaCl and 800 µl (200% of supernatant) of 95% ethanol were also added to the new tube. Genomic DNA was precipitated by leaving it for 1 hour at -80°C. The samples taken out of the -80°C cabinet were kept at room temperature for 5 minutes, and the DNA samples were collected by centrifugation at 13.000 rpm for 10 minutes. Collected DNA samples were washed with 1000 µl of 75% ethanol by centrifugation at 13,000 rpm for 5 minutes, and then ethanol was carefully decanted, and the samples were left to dry. Dried samples were dissolved in 100 µl of nuclease-free water, and after 2 µl of RNAse A was added to each one and incubated for 30 minutes at 37°C, the stock DNA solution was prepared.

Table 4

Stock solutions that are used in DNA Isolation.

Name of Solution	**Contents of Solution**	**Concentrations of Solution**
CTAB Isolation Buffer	Tris-HCl (pH:8.0)	100 mM
	EDTA (pH:8.0)	20 mM
	NaCl	1,4 M
	CTAB(cetyltrimethylammonium bromide)	2%
Chloroform: Octanol	Chloroform	24:1
	Octanol	
Sodium Chloride	NaCl	5M

DNA concentration was measured at 260/280 nm absorbance value using IMPLEN-Nanophotometer P330 model spectrophotometer. According to the results, after the DNA samples were diluted to 50 ng/µl, the remaining samples were stored at -20°C, and the diluted samples were stored at +4°C.

3.1.3. Agarose Gel Electrophoresis

After isolation, 5 µl stock samples are run on a 1% agarose gel to monitor DNA breaks and quality. In the agarose gel preparation, 0.5 g of agarose was dissolved in 50 ml of 1X TAE using a microwave device. After the gel is cooled for 6-7 minutes at room temperature, 3 µl of ethidium bromide is added and mixed, then left on the electrophoresis cassette to polymerize for 15 minutes. In the last part of the gel preparation, the electrophoresis cassette is placed in the electrophoresis tank containing a 1X TAE buffer. During the loading of the samples into the gel, the samples are mixed with 2.5 µl of loading dye and loaded into the gel wells. To run the samples through the gel, the samples were run at 90 volts for 45 minutes, and the gel was examined under UV light using the UVP PhotoDoc-It Imaging System model imaging device to see the results.

Table 5

Solutions that were used for agarose gel electrophoresis.

Chemical Agent	Ingredients
TAE Buffer (10X)	Tris-HCl (pH:8.3)
	EDTA
	Acetic Acid
Loading dye	Bromophenol blue
	Sucrose
Ethidium Bromide	Ethidium Bromide

3.1.4. Detection of SSR Primers

Seven *Rf* genes have been mapped in sunflower (*Helianthus annuus* L.) up to now. Names, linkage groups, and references of *Rf* genes are listed in Table 6. The SSR primers used in the screening study were detected from these mapping studies.

Table 6

Names, linkage groups, and references of *Rf* genes

Gene Name	Linkage Group	Reference
Rf5	LG 13	Qi et al., 2012
Rf1	LG 13	Yue et al., 2010
Rf7	LG 13	Talukder et al., 2019
Rf3	LG 7	Liu et al., 2010
Rf6	LG 3	Liu et al., 2013

Rf-PET-2	LG 13	Sajer et al., 2020
Rf4	LG 3	Feng et al., 2008

21 SSR primers determined to be specific to the *Rf* gene were selected from the mapping studies. The locations of the *Rf* genes and primers in the Linkage groups (LG) are given in Figure 8. Sequence information of SSR primers was obtained from the NCBI database. SSR primer's names and sequences were given in Appendix 2.

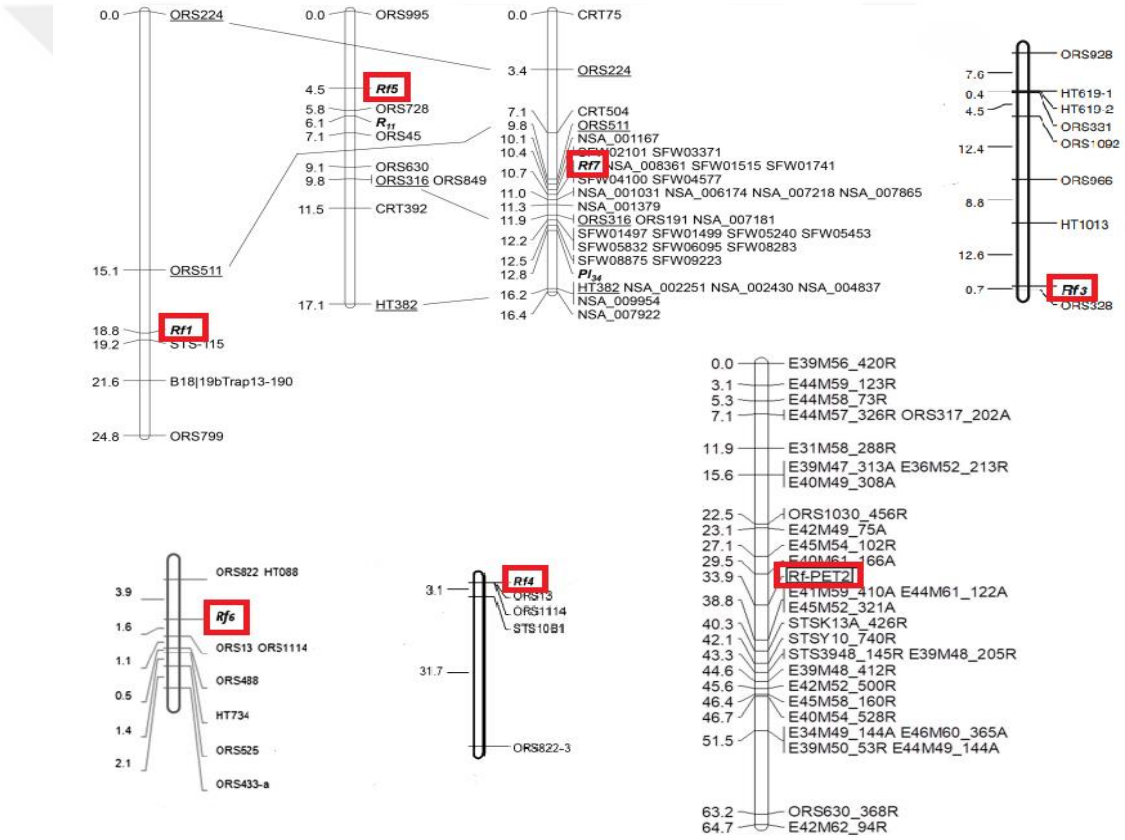


Figure 8. Mapped *Rf* genes

3.1.5. Bulk Segregant Analysis (BSA)

The bulk segregant analysis method was used to understand the general polymorphism of these selected markers in our interest in commercial sunflower genotypes. BSA method was applied to parental individuals that we know to be sterile or non-sterile. Four DNA pools of 300 µl were obtained by adding 10 µl of each DNA sample, which was previously adjusted to 50 ng/ml and stored at +4°C. The preparation of DNA pools for bulk segregation analysis was summarized in Figure 9.

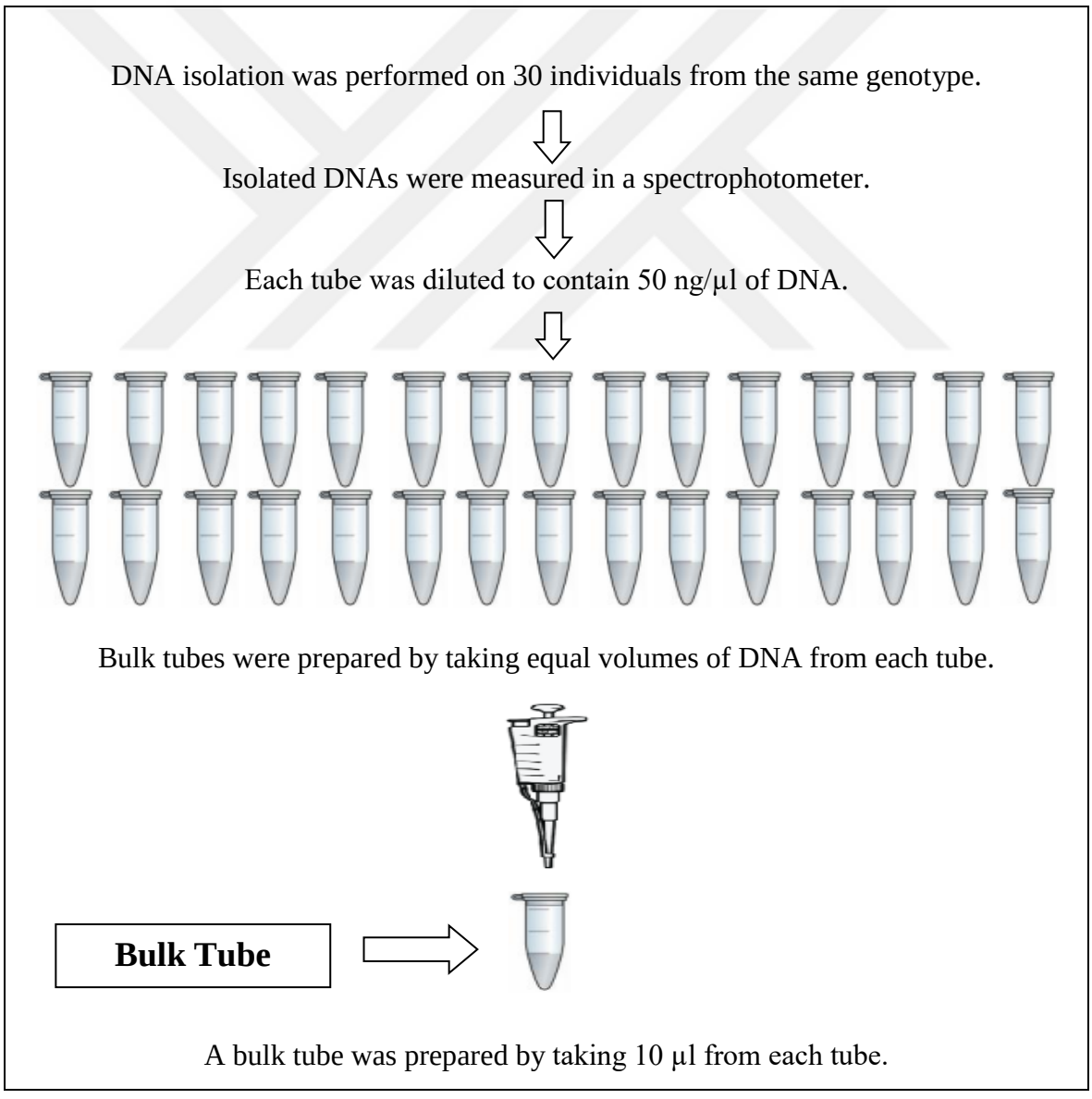


Figure 9. Preparation of DNA pools

3.1.6. Polymerase Chain Reaction (PCR)

PCR was performed using 21 selected SSR primers on 4 DNA pools of parental individuals obtained by the BSA method. Volume and concentrations of PCR components are given in Table 7, and the PCR program used in the thermal cycler for SSR primers is given in Table 8. DNA pools were screened with these SSR primers to detect polymorphism. The screening studies were performed on F_2 individuals with the 5 SSR primers, which was found to form polymorphic band profile between DNA pools. The names and sequences of the SSR primers found to create polymorphic band profiles between the DNA pools are given in Table 9.

Table 7

PCR components.

PCR Components	**Concentration**	**Volume (µl)**
10 X Taq Buffer	10 X	2
MgCl2	50 mM	1
dNTP	2.5 mM	0,4
SSR Forward Primer	10 µM	1
SSR Reverse Primer	10 µM	1
Taq DNA Polymerase	5 U/µl	0,1
Genomic DNA	50 ng/µl	2
dH2O	-	12,5

Table 8

PCR program that was used in thermal cycler for SSR primers.

Reaction Step	Number of Cycle	Temperature (°C)	Time (min)
Initial Denaturation	1	94°C	03:00
Denaturation	40	94°C	01:00
Annealing Temperature		48-54°C	01:00
Extension		72°C	01:00
Final Extension	1	72°C	10:00
Hold	1	+4°C	∞

Table 9

SSR primers that form polymorphic band structures.

Name of SSR Primer	Forward Primer	Reverse Primer
ORS 511	TggCTCAgATTAAgTTCACACAg	CgggTTgCgAgTAACAggTA
ORS 630	gCACgACCCggATATgTAAC	TgTgCTgAggATgATATgCAg
ORS 822	CAATgCCATCTgTCATCAgCTAC	AAACAAACCTTTggACgAAACTC
ORS 928	CATggTTATTTTggTTTgggTTT	gCTATTATCATgTCCTTgTCCTTTT
ORS 995	CATgCTTTCTAggATggTCAgTT	TgTATgTggAggCCAACAAgTAT

CHAPTER 4
RESEARCH FINDINGS

4.1. Genomic DNA Isolation

DNA isolation of a total of 120 individuals from four different sunflowers (*Helianthus annuus* L.) genotypes that are parental individuals and a total of 200 individuals from two different F_2 cultivars was performed according to the CTAB-based genomic DNA isolation protocol of Doyle and Doyle (1987). Before starting the polymorphism screening studies, DNA qualities were examined using agarose gel electrophoresis, as shown in Figure 10. DNA concentrations were also measured using the IMPLEN-Nanophotometer P330 model spectrophotometer.

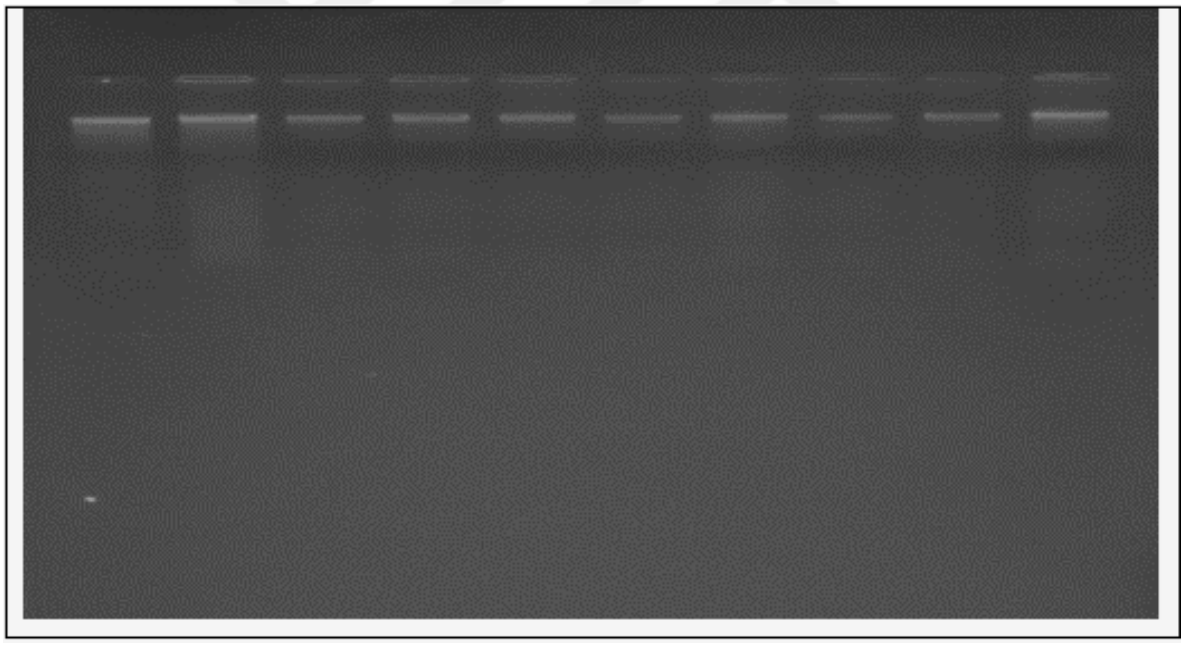


Figure 10. Sample image of DNA isolation results viewed with 1% agarose gel

4.2. Bulk Segregant Analysis

4.2.1. Screening of Bulk Tubes

21 of the SSR primers that are specific to *Rf* genes were detected for this screening study, and four DNA pools were screened with these primers. 2% agarose gel was used to detect the polymorphism between the products formed due to PCR. SSR primers giving monomorphic and polymorphic band profiles after screening are listed in Table 10.

Table 10
SSR primers giving monomorphic and polymorphic band profiles

Primers Showing Monomorphic Band Structure	Primers Showing Polymorphic Band Structure
ORS 13, ORS 45, ORS 191, ORS 224, ORS 316, ORS 317, ORS 328, ORS 331, ORS 488, ORS 728, ORS 799, ORS 849, ORS 966, ORS 1030, ORS 1092, ORS 1114	ORS 511, ORS 630, ORS 822, ORS 928, ORS 995

Figure 11 is given as an example of monomorphic and polymorphic results. As seen in the image, the bulks were screened with ORS 966 SSR primer, and it was determined that they formed a monomorphic band structure since there was no difference between the band profiles. For this reason, the screening result with ORS 966 SSR primer does not give a meaningful result for detecting individuals carrying the *Rf* gene. However, when the bulks were screened with the ORS 630 SSR primer, a distinctly different band profile was observed among the PCR product, indicated by the number 8 in the image. Screening result 8 represents the bulk of the 3814-R genotype in the screening study that was performed with the ORS 630 SSR primer, a polymorphic band profile was formed in the 3814-R genotype. Therefore, the ORS 630 SSR primer was selective for the 3814-R genotype individuals. In addition, the screening results of F_2 individuals were required for this primer to be usable in the marker-assisted selection method. For this reason, SSR primers, found to form

polymorphic band structure in bulk screening, were scanned for individuals forming the bulk and F_2 individuals.

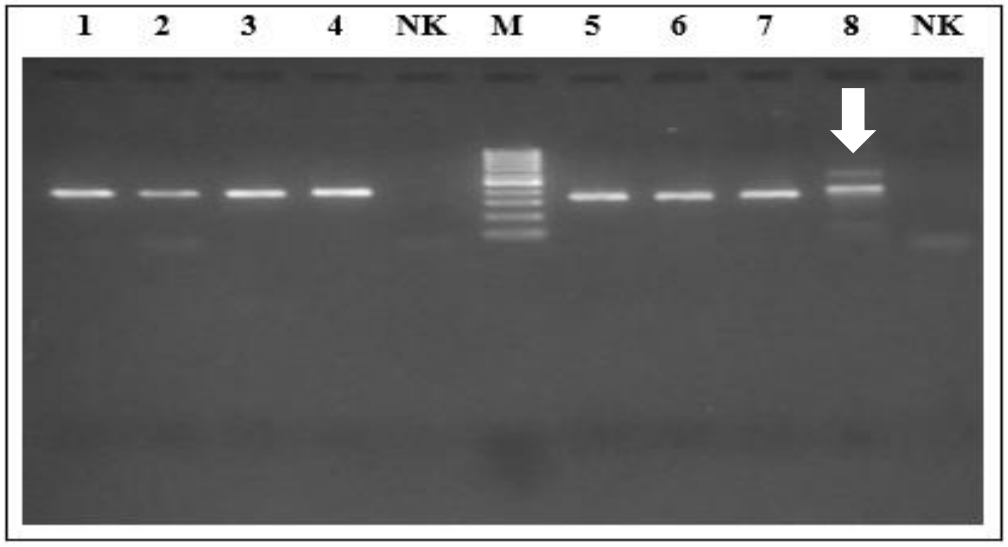


Figure 11. This image shows the PCR products of bulks of sunflower (*H. annuus* L.) genotypes obtained with ORS 966 and ORS 630 SSR marker in 2% agarose gel. **1:** 9728-A (ORS 966 marker), **2:** CL-217-R (ORS 966 marker), **3:** IMI-044-A (ORS 966 marker), **4:** 3814-R (ORS 966 marker), **5:** 9728-A (ORS 630 marker), **6:** CL-217-R (ORS 630 marker), **7:** IMI-044-A (ORS 630 marker), **8:** 3814-R (ORS 630 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50bp DNA ladder

4.3. Individual Screening of SSR Primers Producing Polymorphism

4.3.1. Screening with ORS 995 SSR Primer

As a result of bulk screening with ORS 995 SSR primer, polymorphism between the formed band profiles was observed. The polymorphism results are given in Figure 12. The lengths of the bands formed as a result of screening are shown in Table 11. It was observed that the sizes of the PCR products of the CL-217-R and 3814-R genotypes, which were known to carry *Rf* gene, were similar to each other. Both of them have a 150 bp band length. And also, it was observed that the sizes of the PCR products of the 9728-A and IMI-044-A genotypes that didn't carry *Rf* gene were similar. Both of them have 135 bp band lengths. However, there was a difference between the PCR products of the genotypes that were

known to carry *Rf* gene and the PCR products of the genotypes that were known don't carry *Rf* gene, and this caused polymorphism.

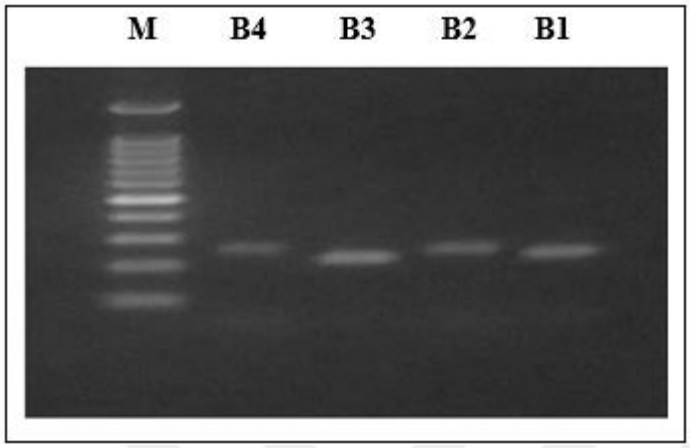


Figure 12. This image shows the PCR products of sunflower (*H. annuus* L.*)* genotypes bulks obtained with ORS 995 SSR marker in 2% agarose gel.
B1: 9728-A bulk (ORS 995 marker), **B2:** CL-217-R bulk (ORS 995 marker), **B3:** IMI-044-A bulk (ORS 995 marker), **B4:** 3814-R bulk (ORS 995 marker), **M:** Marker, GeneRuler 50 bp DNA ladder

Table 11
Evaluation results of band profiles formed after screening with ORS 995 SSR primer by Logger Pro 3.16.2

Name of Genotype	**9728-A**	**CL-217-R**	**IMI-044-A**	**3814-R**
Band Length	135 bp	150 bp	135 bp	150bp

To observe the polymorphism formed as a result of bulk screening with ORS 995 in individuals and to determine how many of the individuals forming the bulk gave similar results with the bulk, the ORS 995 screening of the individuals forming the bulk were also performed. The screening results of bulk and individuals of 9728-A genotype were given in Figure 13, screening results of bulk and individuals of CL-217-R genotype in Figure 14, screening results of bulk and individuals of IMI-044-A genotype in Figure 15, and screening results of bulk and individuals of the 3814-R genotype in Figure 16.

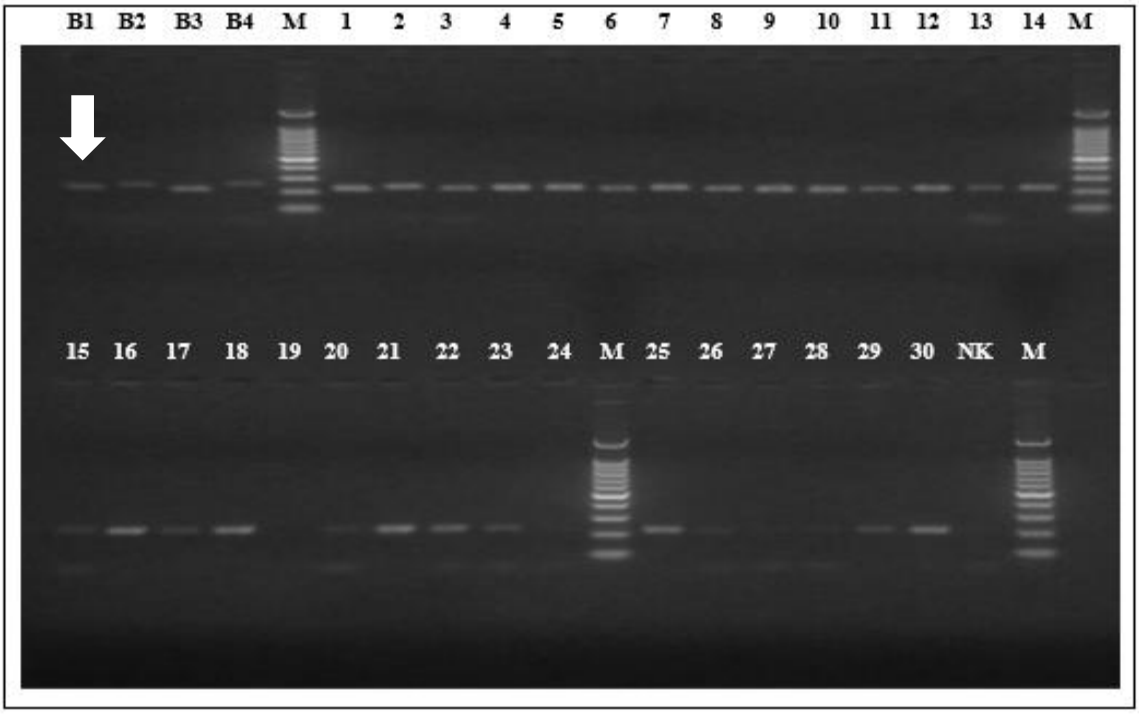


Figure 13**.** This image shows the PCR products of sunflower bulks and individuals of 9728-A genotypes obtained with ORS 995 SSR marker in 2% agarose gel.
B1: 9728-A bulk (ORS 995 marker), **B2:** CL-217-R bulk (ORS 995 marker), **B3:** IMI-044-A bulk (ORS 995 marker), **B4:** 3814-R bulk (ORS 995 marker), **1-30:** Individuals of 9728-A genotypes (ORS 995 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

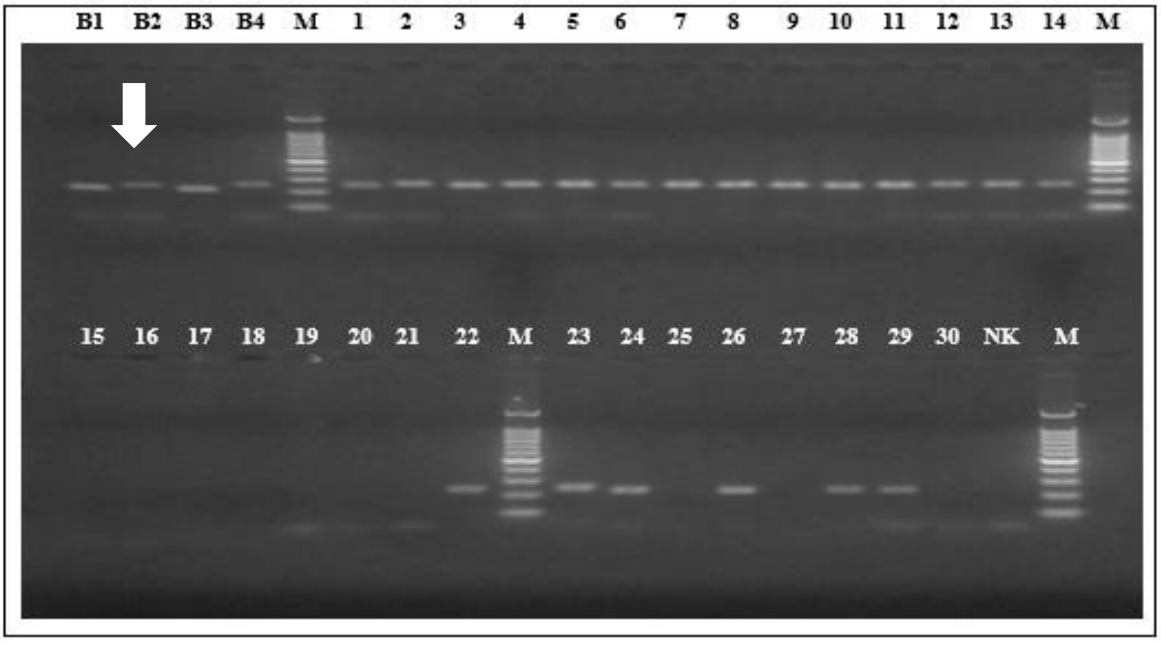


Figure 14. This image shows the PCR products of sunflower bulks and individuals of CL-217-R genotypes obtained with ORS 995 SSR marker in 2% agarose gel.
B1: 9728-A bulk (ORS 995 marker), **B2:** CL-217-R bulk (ORS 995 marker), **B3:** IMI-044-A bulk (ORS 995 marker), **B4:** 3814-R bulk (ORS 995 marker), **1-30:** Individuals of CL-

217-R genotypes (ORS 995 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

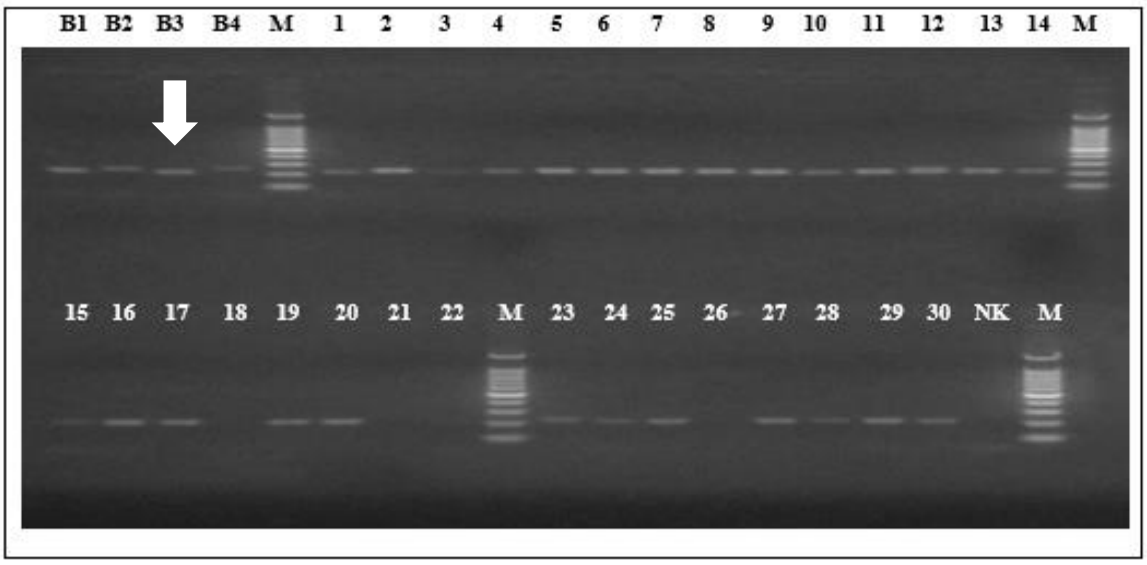


Figure 15. This image shows the PCR products of sunflower bulks and individuals of IMI-044-A genotypes obtained with ORS 995 SSR marker in 2% agarose gel.
B1: 9728-A bulk (ORS 995 marker), **B2:** CL-217-R bulk (ORS 995 marker), **B3:** IMI-044-A bulk (ORS 995 marker), **B4:** 3814-R bulk (ORS 995 marker), **1-30:** Individuals of IMI-044-A genotypes (ORS 995 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

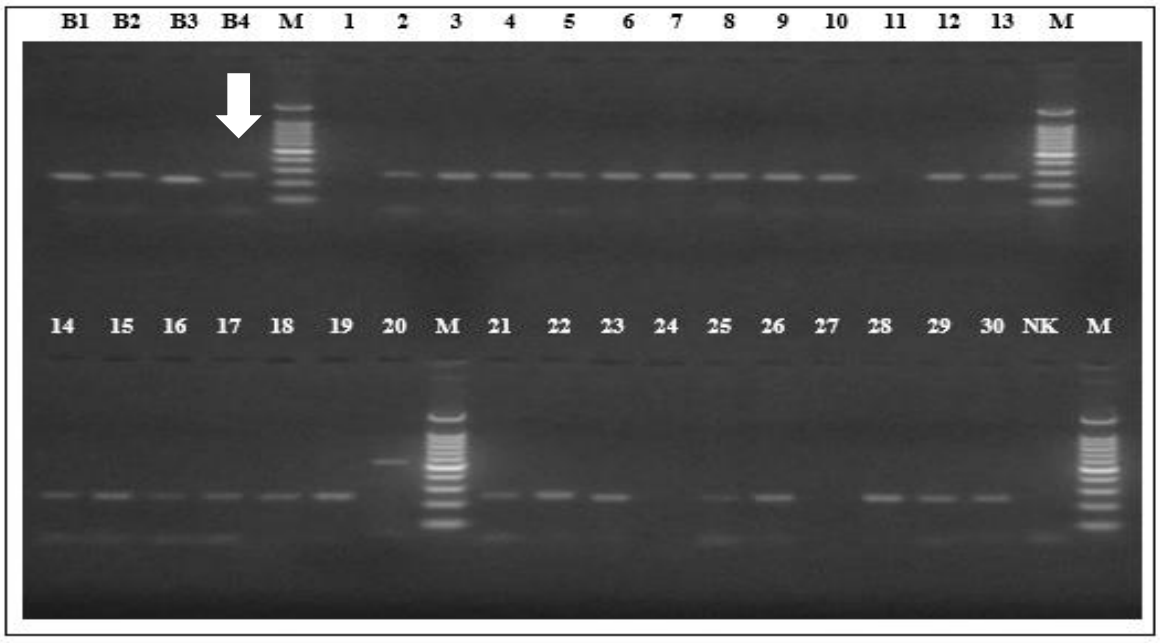


Figure 16. This image shows the PCR products of sunflower bulks and individuals of 3814-R genotypes obtained with ORS 995 SSR marker in 2% agarose gel.
B1: 9728-A bulk (ORS 995 marker), **B2:** CL-217-R bulk (ORS 995 marker), **B3:** IMI-044-A bulk (ORS 995 marker), **B4:** 3814-R bulk (ORS 995 marker), **1-30:** Individuals of 3814-

R genotypes (ORS 995 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

After the screening of parental individuals, F_2 individuals were screened. Two kinds of F_2 individuals were used. Parental individuals with 9728-A genotype and CL-217-R genotypes were used to obtain one variety, while parent individuals with IMI-044-A genotype and 3814-R genotype were used to obtain the other F_2 variety. While creating individuals of both F_2 cultivars, parental genotype cultivars carrying the *Rf* gene and not carrying the *Rf* gene were used. Therefore, for the ORS 995 SSR primer, which creates a different band profile between carrying the *Rf* gene and not carrying the *Rf* gene, screening results of F_2 individuals give a lot of information about the usability of the primer in the MAS method.

The screening results of F_2 individuals created using 9728-A genotype, and CL-217-R genotypes were given in Figure 17.

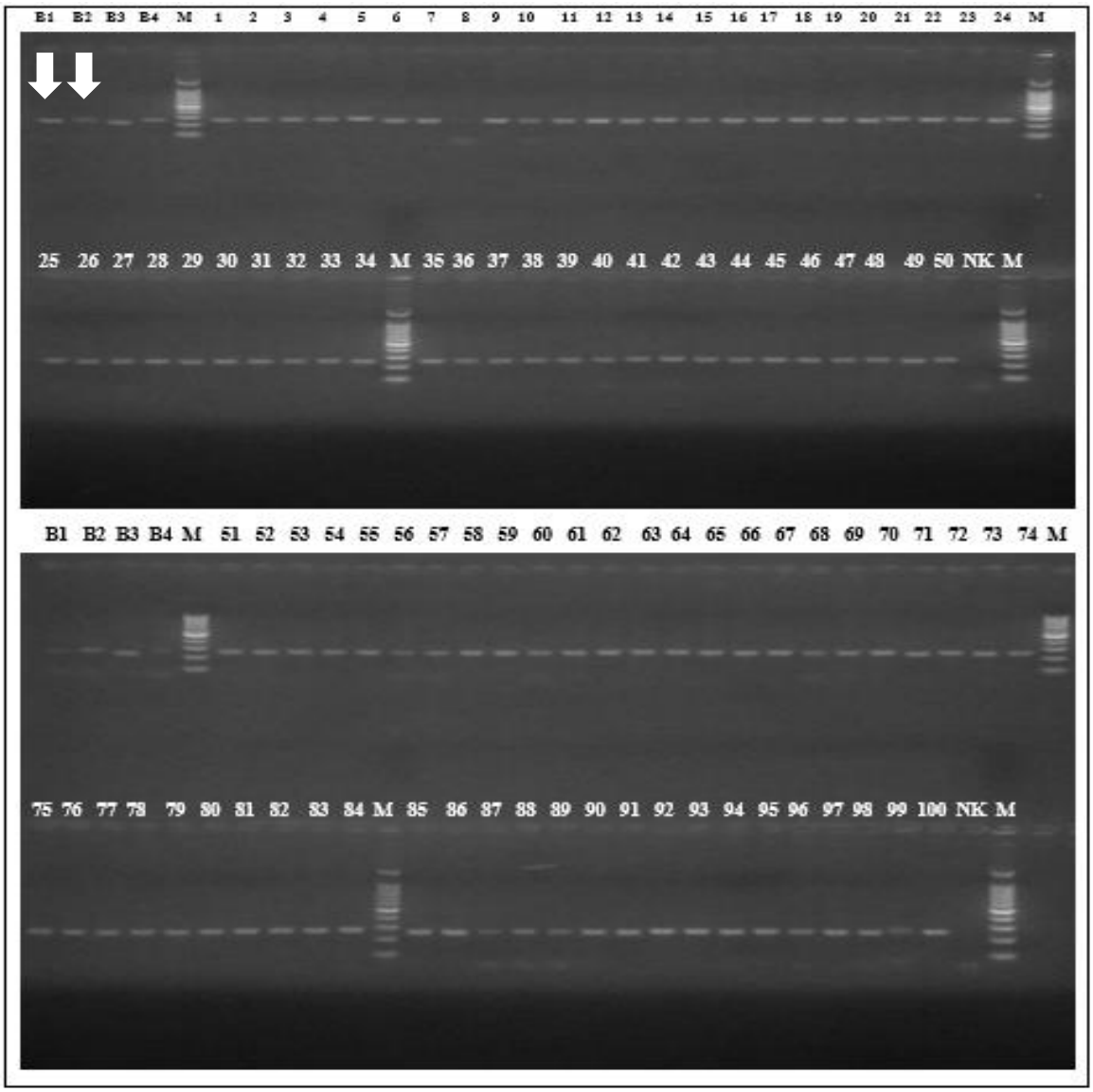


Figure 17. This image shows the PCR products of sunflower bulks and F_2 individuals formed by crossing 9728-A x CL-217-R genotypes obtained with ORS 995 SSR marker in 2% agarose gel.
B1: 9728-A bulk (ORS 995 marker), **B2:** CL-217-R bulk (ORS 995 marker), **B3:** IMI-044-A bulk (ORS 995 marker), **B4:** 3814-R bulk (ORS 995 marker), **1-100:** F_2 individuals (ORS 995 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

The screening results of F_2 individuals created using the IMI-044-A genotype, and 3814-R genotypes were given in Figure 18.

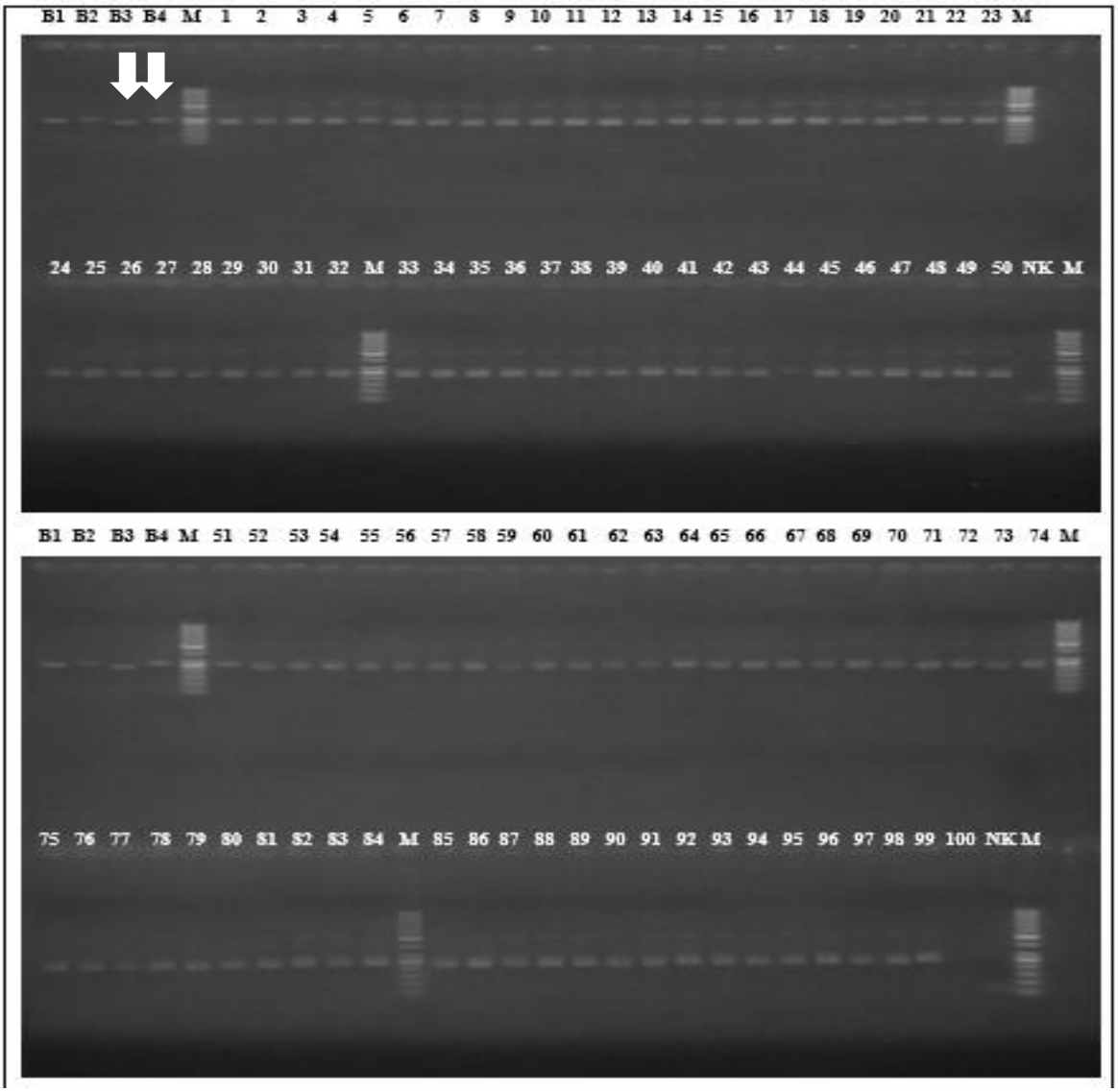


Figure 18. This image shows the PCR products of sunflower bulks and F_2 individuals formed by crossing IMI-044-A x 3814-R genotypes obtained with ORS 995 SSR marker in 2% agarose gel.

B1: 9728-A bulk (ORS 995 marker), **B2:** CL-217-R bulk (ORS 995 marker), **B3:** IMI-044-A bulk (ORS 995 marker), **B4:** 3814-R bulk (ORS 995 marker), **1-100:** F_2 individuals (ORS 995 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

One hundred individuals from both F_2 cultivars were screened. ORS 995 SSR primer was formed 150 bp band in the screening F_2 individuals that were created using the 9728-A genotype, and CL-217-R genotypes, and the same band profile was observed with the CL-217-R genotype that were known to carry *Rf* gene. It was observed that 97 out of 100 F_2 individuals formed by using the IMI-044-A genotype and 3814-R genotype formed two

bands of 135 bp and 150 bp and carried band profiles of both IMI-044-A genotype and 3814-R genotype.

4.3.2. Screening with ORS 511 SSR Primer

As a result of bulk screening with ORS 511 SSR primer, polymorphism between the formed band profiles was observed. DNA pool screening result was given in Figure 19. The lengths of the bands formed as a result of scanning are shown in Table 12. In the screening result of DNA pools, ORS 511 SSR primer was created band profile only with genotypes that were known to carry *Rf* gene. Therefore this situation caused polymorphism.

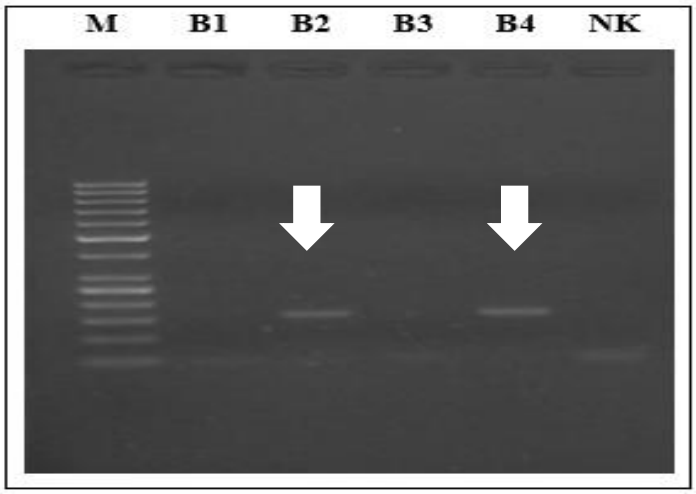


Figure 19. This image shows the PCR products of sunflower (*H. annuus* L.) genotypes bulks obtained with ORS 511 SSR marker in 2% agarose gel.
B1: 9728-A bulk (ORS 511 marker), **B2:** CL-217-R bulk (ORS 511 marker), **B3:** IMI-044-A bulk (ORS 511 marker), **B4:** 3814-R bulk (ORS 511 marker), **M:** Marker, GeneRuler 50 bp DNA ladder, **NK:** Negative Control

Table 12
Evaluation results of bands formed after screening with ORS 511 SSR primer by Logger Pro 3.16.2

Name of Genotype	**9728-A**	**CL-217-R**	**IMI-044-A**	**3814-R**
Band Length	-	180 bp	-	180 bp

As a result of bulk scanning with ORS 511, a single variant with a length of 180 bp was formed. To observe this band polymorphism in individuals and determine how many individuals forming the bulk give similar results to their own bulk, the ORS 511 screening of the individuals forming the bulk were also performed. The screening results of bulk and individuals of 9728-A genotype were given in Figure 20, screening results of bulk and individuals of CL-217-R genotype in Figure 21, screening results of bulk and individuals of IMI-044-A genotype in Figure 22, and screening results of bulk and individuals of 3814-R genotype in Figure 23.

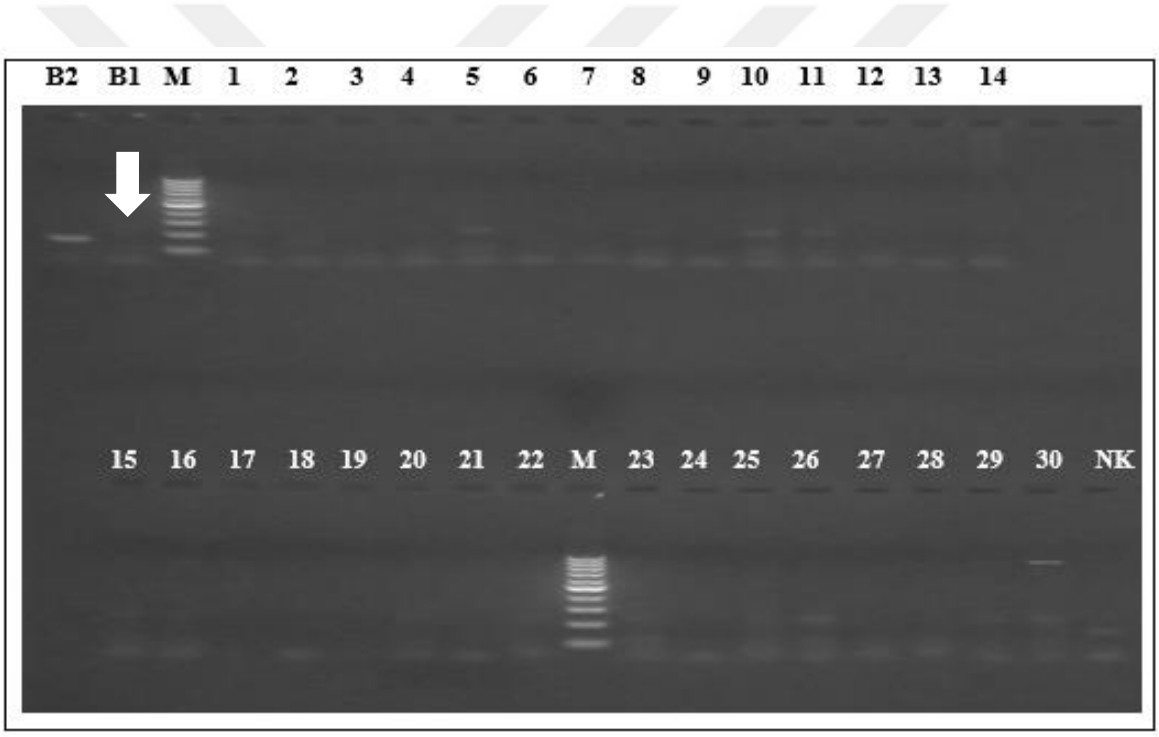


Figure 20. This image shows the PCR products of sunflower bulks and individuals of 9728-A genotypes obtained with ORS 511 SSR marker in 2% agarose gel.
B2: CL-217-R bulk (ORS 511 marker), **B1:** 9728-A bulk (ORS 511 marker), **1-30:** Individuals of 9728-A genotypes (ORS 511 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

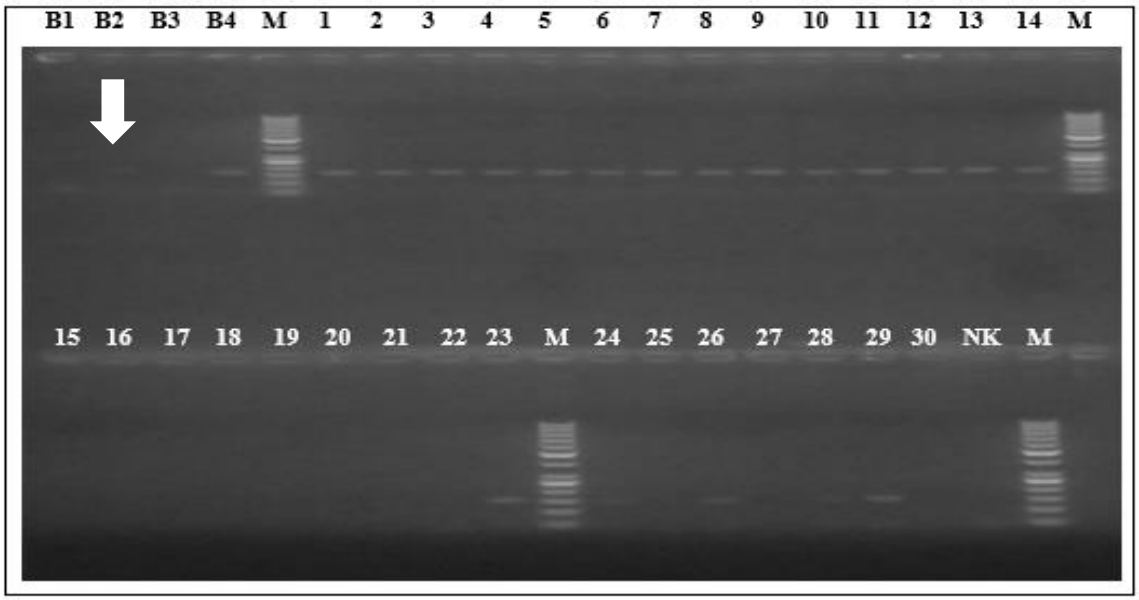


Figure 21. This image shows the PCR products of sunflower bulks and individuals of CL-217-R genotypes obtained with ORS 511 SSR marker in 2% agarose gel.
B1: 9728-A bulk (ORS 511 marker), **B2:** CL-217-R bulk (ORS 511 marker), **B3:** IMI-044-A bulk (ORS 511 marker), **B4:** 3814-R bulk (ORS 511 marker), **1-30:** Individuals of CL-217-R genotypes (ORS 511 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

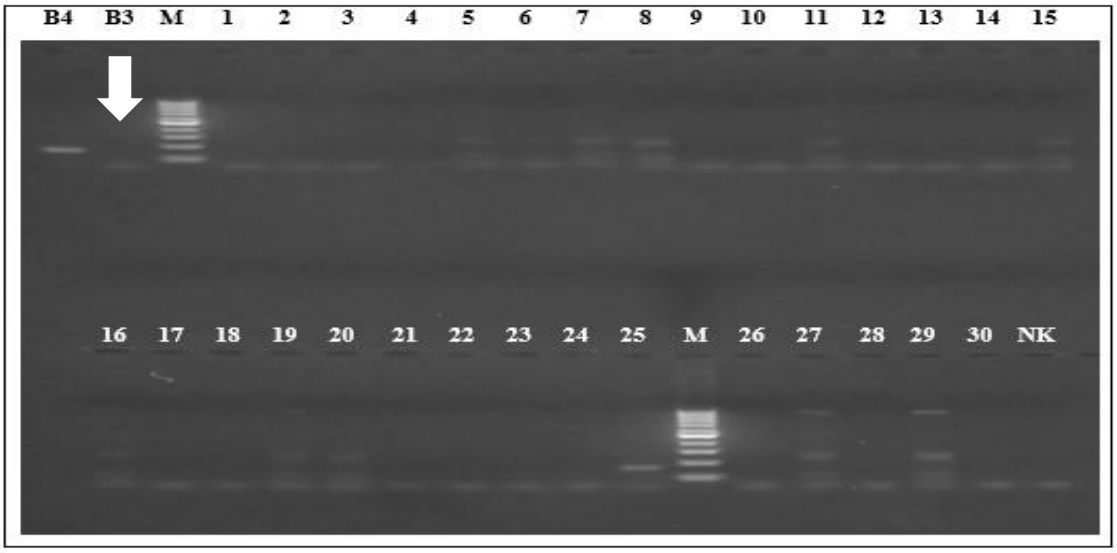


Figure 22. This image shows the PCR products of sunflower bulks and individuals of IMI-044-A genotypes obtained with ORS 511 SSR marker in 2% agarose gel.
B4: 3814-R bulk (ORS 511 marker), **B3:** IMI-044-A bulk (ORS 511 marker), **1-30:** Individuals of IMI-044-A genotypes (ORS 511 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

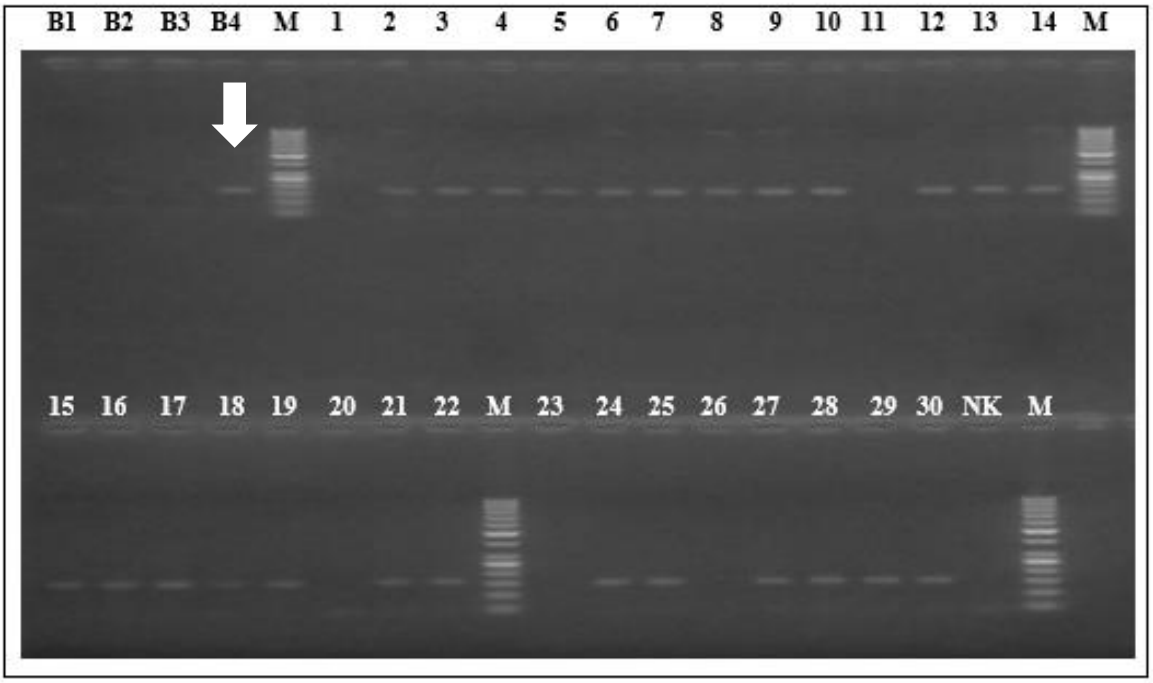


Figure 23. This image shows the PCR products of sunflower bulks and individuals of 3814-R genotypes obtained with ORS 511 SSR marker in 2% agarose gel.
B1: 9728-A bulk (ORS 511 marker), **B2:** CL-217-R bulk (ORS 511 marker), **B3:** IMI-044-A bulk (ORS 511 marker), **B4:** 3814-R bulk (ORS 511 marker), **1-30:** Individuals of 3814-R genotypes (ORS 511 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

The screening results of F_2 individuals created using 9728-A genotype and CL-217-R genotypes were given in Figure 24.

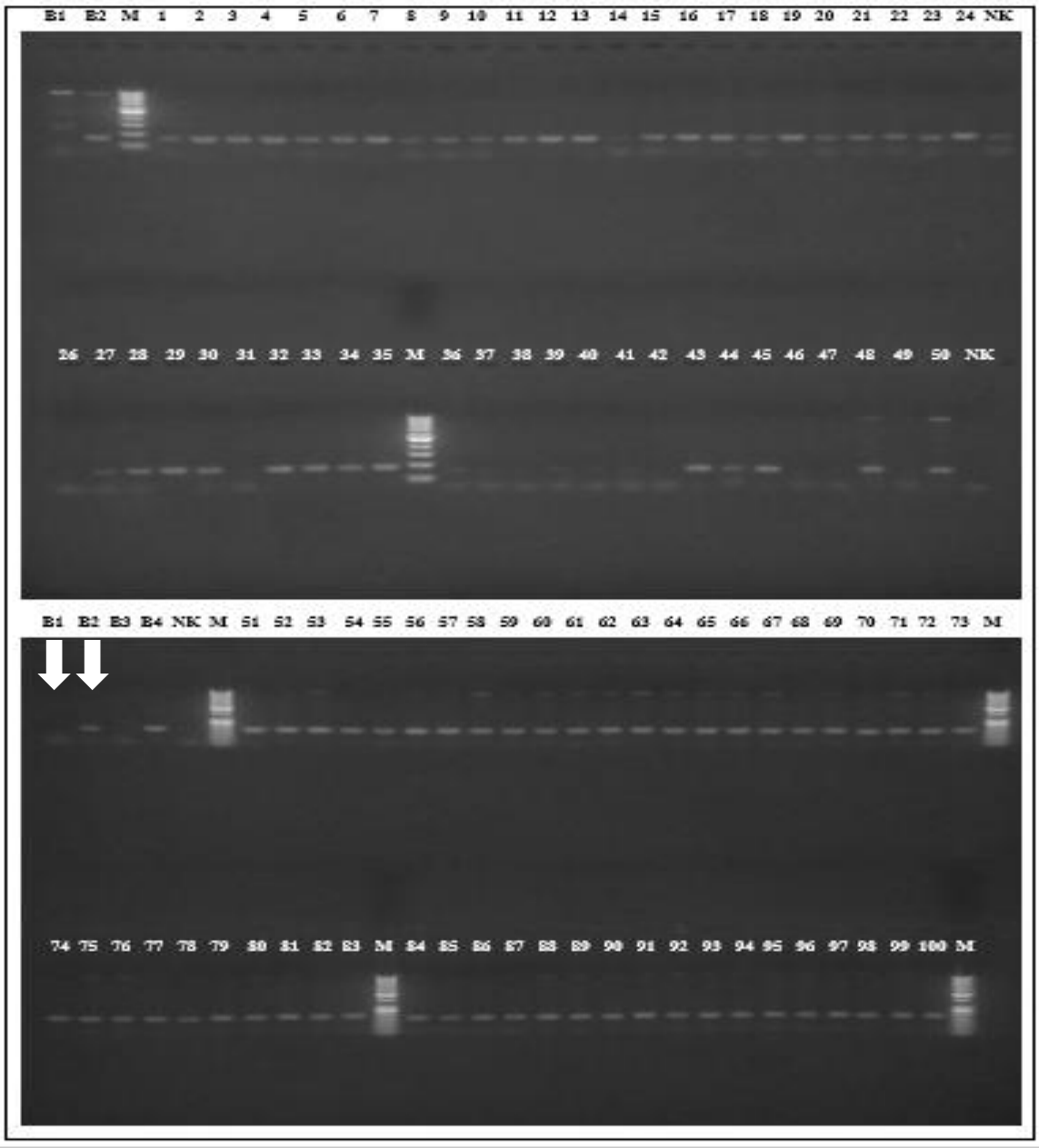


Figure 24. This image shows the PCR products of sunflower bulks and F_2 individuals formed by crossing 9728-A x CL-217-R genotypes obtained with ORS 511 SSR marker in 2% agarose gel.
B1: 9728-A bulk (ORS 511 marker), **B2:** CL-217-R bulk (ORS 511 marker), **B3:** IMI-044-A bulk (ORS 511 marker), **B4:** 3814-R bulk (ORS 511 marker), **1-100:** F_2 individuals (ORS 511 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

The screening results of F_2 individuals created using the IMI-044-A genotype and 3814-R genotypes were given in Figure 25.

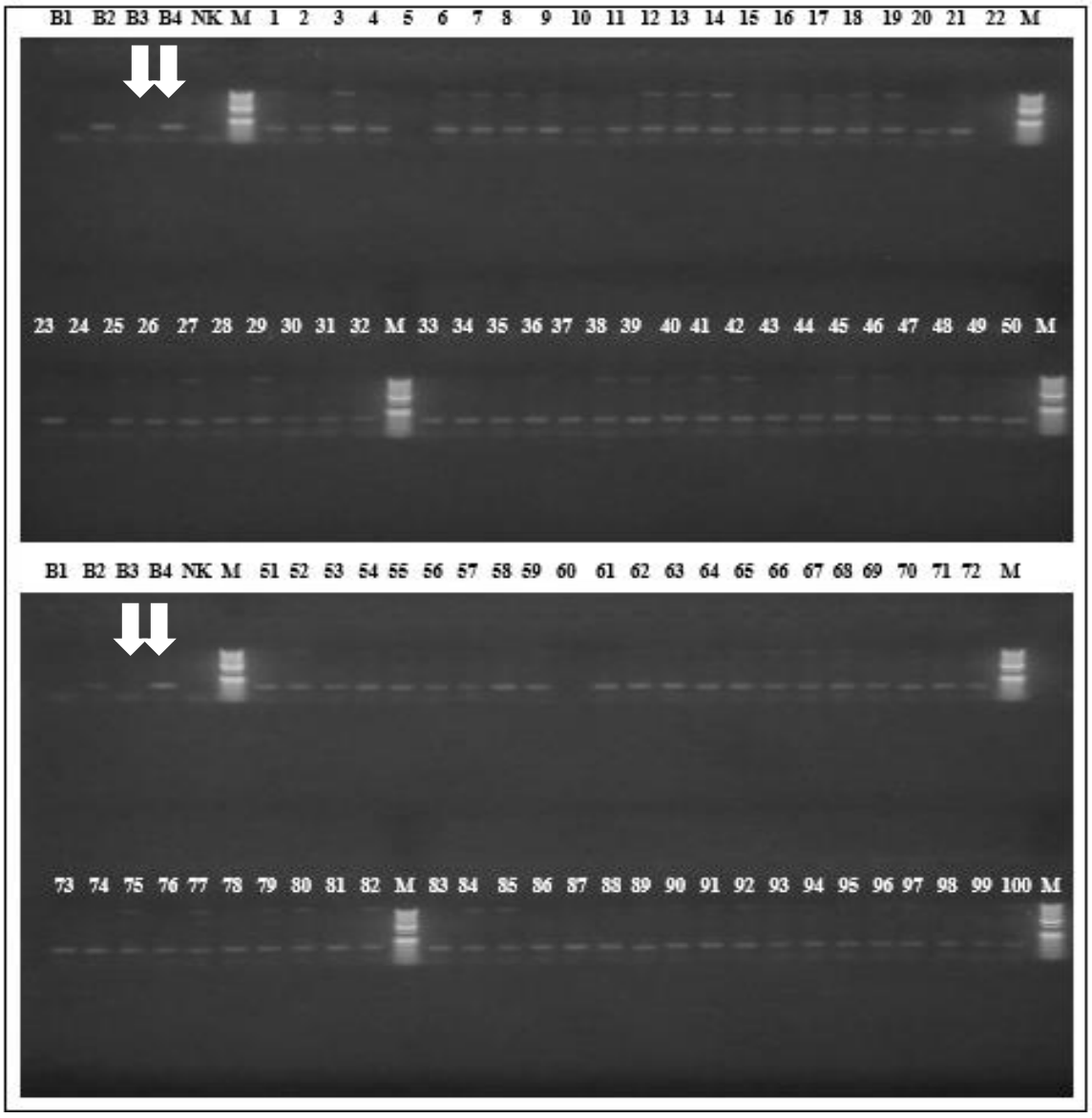


Figure 25. This image shows the PCR products of sunflower bulks and F_2 individuals formed by crossing IMI-044-A x 3814-R genotypes obtained with ORS 511 SSR marker in 2% agarose gel.
B1: 9728-A bulk (ORS 511 marker), **B2:** CL-217-R bulk (ORS 511 marker), **B3:** IMI-044-A bulk (ORS 511 marker), **B4:** 3814-R bulk (ORS 511 marker), **1-100:** F_2 individuals (ORS 511 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

As a result of screening of parental individuals, 180 bp band formation is seen only in individuals carrying the *Rf* gene. This band formation was observed in 87 out of 100 F_2 individuals created using 9728-A and CL-217-R genotypes. This band formation was observed in 97 out of 100 F_2 individuals, which were formed by crossing the IMI-044-A genotype and 3814-R genotype.

4.3.3. Screening with ORS 822 SSR Primer

As a result of bulk screening with ORS 822 SSR primer, polymorphism was detected between the formed band profiles. DNA pool screening results were given in Figure 26. As a result of the screening, two band profiles with a length of 170 bp and 80 bp were observed in the bulk of the 9728-A, CL-217-R, and IMI-044-A genotypes, while a single band profile of 80 bp in length was observed in the bulk screening of the 3814-R genotype and this situation created polymorphism. This situation led us to think that the ORS 822 SSR primer could be selective for the 3814-R genotype and that marker-assisted selection could be used for this genotype carrying the *Rf* gene.

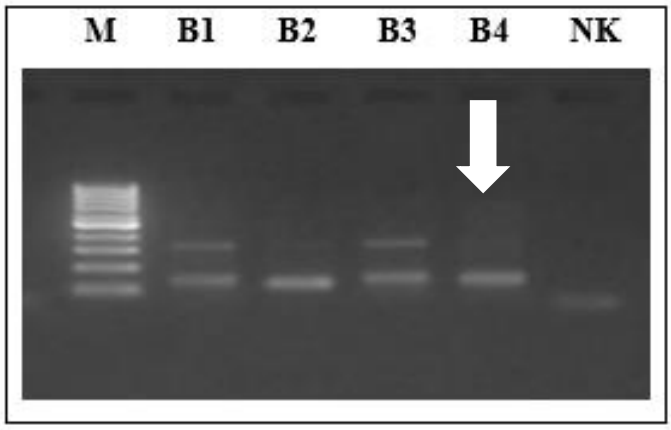


Figure 26. This image shows the PCR products of sunflower (*H. annuus* L.) genotypes bulks obtained with ORS 822 SSR marker in 2% agarose gel.
B1: 9728-A bulk (ORS 822 marker), **B2:** CL-217-R bulk (ORS 822 marker), **B3:** IMI-044-A bulk (ORS 822 marker), **B4:** 3814-R bulk (ORS 822 marker), **M:** Marker, GeneRuler 50 bp DNA ladder, **NK:** Negative Control

Table 13
Evaluation results of bands formed after screening with ORS 822 SSR primer by Logger Pro 3.16.2

Name of Genotype	9728-A	CL-217-R	IMI-044-A	3814-R
First Band	170 bp	170 bp	170 bp	-
Second Band	80 bp	80 bp	80 bp	80 bp

To observe the polymorphism formed as a result of bulk screening with ORS 822 in individuals and to determine how many of the individuals that formed the bulk gave similar results with their own bulk. Since 9728-A, CL-217-R, and IMI-044-A genotypes show a monomorphic band profile among themselves, it was considered sufficient to look at only one of the individuals forming the bulk. For this reason, only bulk individuals of IMI-044-A and 3814-R genotypes were screened. The screening results of the bulk and individuals of the IMI-044-A genotype were given in Figure 27, and the screening results of the bulk and individuals of the 3814-R genotype in Figure 28.

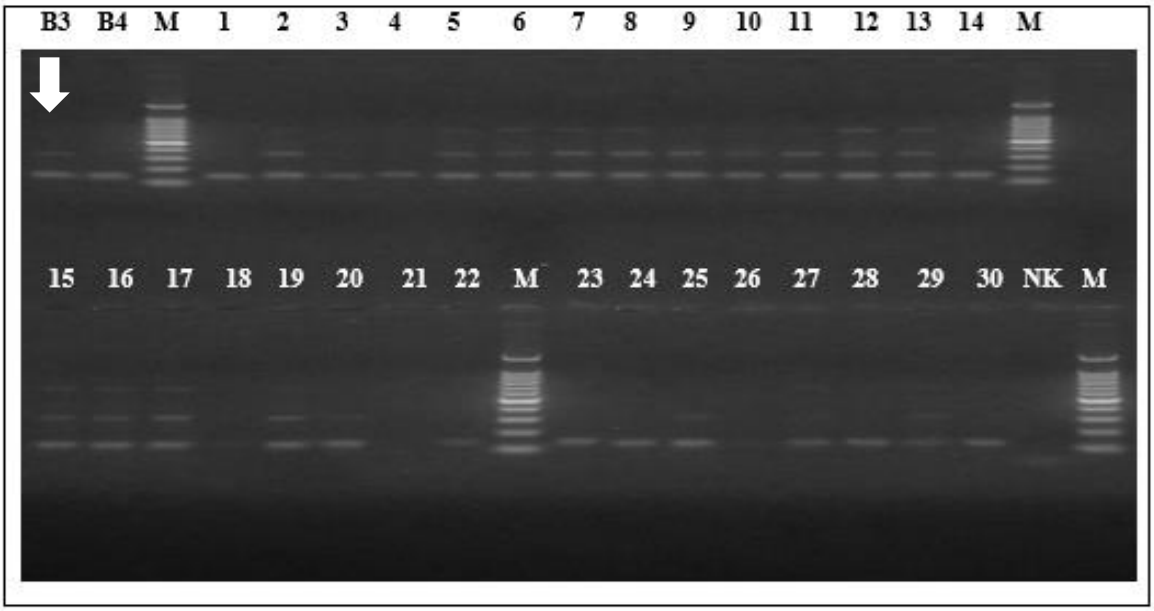


Figure 27. This image shows the PCR products of sunflower bulks and individuals of IMI-044-A genotypes obtained with ORS 822 SSR marker in 2% agarose gel.
B3: IMI-044-A bulk (ORS 822 marker), **B4:** 3814-R bulk (ORS 822 marker), **1-30:** Individuals of IMI-044-A genotypes (ORS 822 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

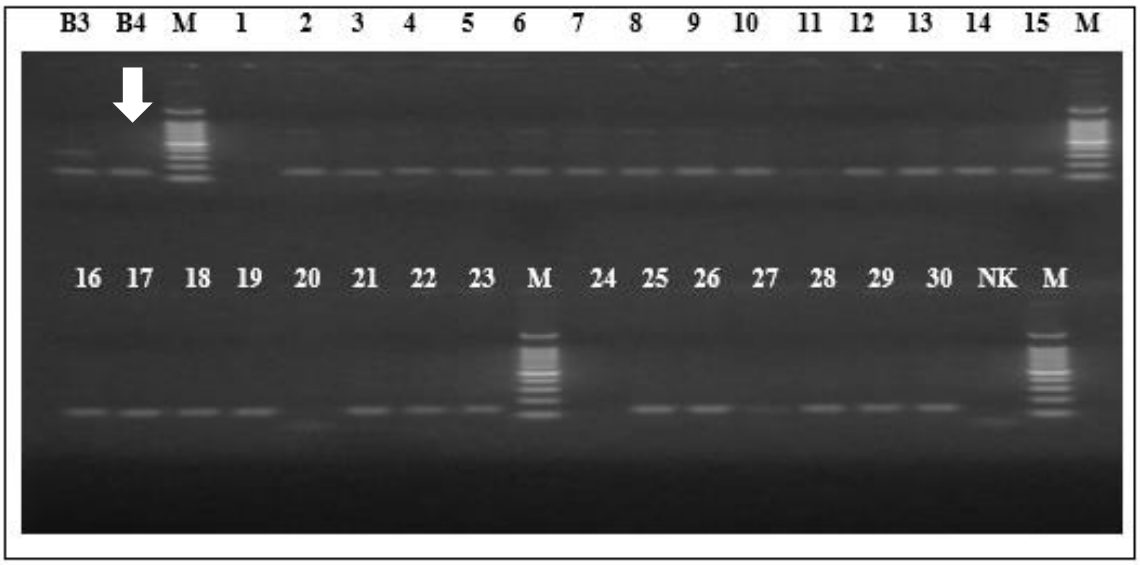


Figure 28. This image shows the PCR products of sunflower bulks and individuals of 3814-R genotypes obtained with ORS 822 SSR marker in 2% agarose gel.
B3: IMI-044-A bulk (ORS 822 marker), **B4:** 3814-R bulk (ORS 822 marker), **1-30:** Individuals of 3814-R genotypes (ORS 822 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

The screening results of F_2 individuals created using the IMI-044-A genotype and 3814-R genotypes were given in Figure 29.

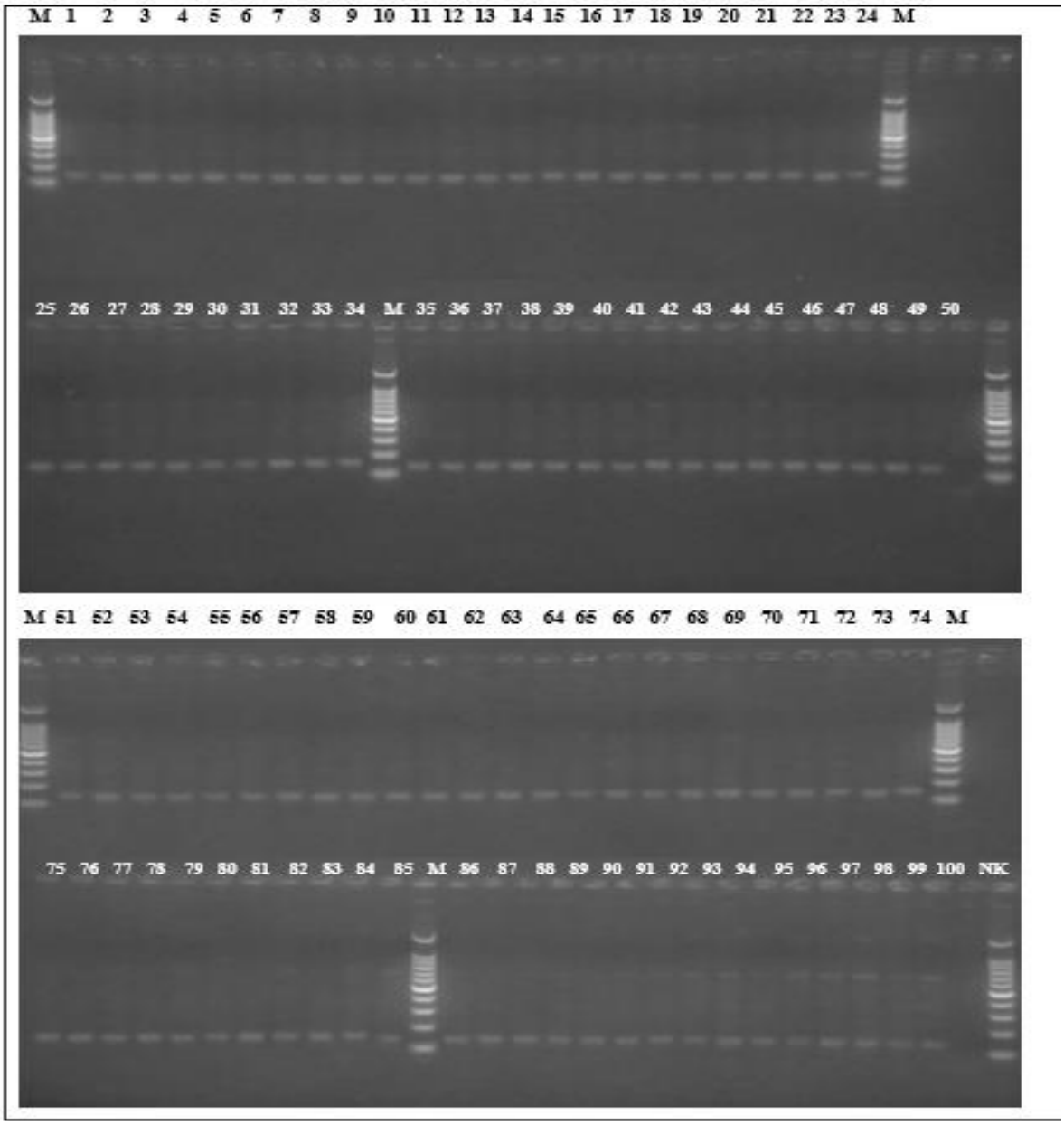


Figure 29. This image shows the PCR products of sunflower bulks and F_2 individuals formed by crossing IMI-044-A x 3814-R genotypes obtained with ORS 822 SSR marker in 2% agarose gel.
1-100: F_2 individuals (ORS 822 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

100 F_2 individuals were created using the IMI-044-A genotype, and 3814-R genotypes were screened with ORS 822 SSR primer. During the bulk screening, two bands of 80 bp and 170 bp lengths were observed in the bulks of the 9728-A, CL-217-R, and IMI-044-A genotypes, while a single band formed only 80 bp in length was observed in the 3814-

R genotype. When F_2 individuals were screened, only an 80 bp long band of PCR product was observed in F_2 individuals created using the IMI-044-A and 3814-R genotypes.

4.3.4. Screening with ORS 630 SSR Primer

As a result of bulk screening with ORS 630 SSR primer, polymorphism between band profiles was observed. DNA pool screening result was given in Figure 30. As a result of the screening, a single band profile was observed in the bulk of the 9728-A, CL-217-R, and IMI-044-A genotypes, while a double band profile was observed in the bulk screening of the 3814-R genotype and created polymorphism. The lengths of the PCR products forming the bands are given in Table 14. This situation led us to think that the ORS 630 SSR primer could be selective for the 3814-R genotype and that marker-assisted selection could be used for this genotype carrying the *Rf* gene.

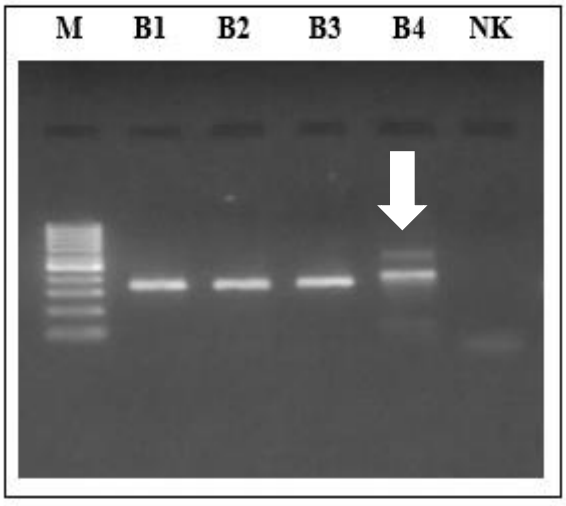


Figure 30. This image shows the PCR products of sunflower (*H. annuus* L.) genotypes bulks obtained with ORS 630 SSR marker in 2% agarose gel.
B1: 9728-A bulk (ORS 630 marker), **B2:** CL-217-R bulk (ORS 630 marker), **B3:** IMI-044-A bulk (ORS 630 marker), **B4:** 3814-R bulk (ORS 630 marker), **M:** Marker, GeneRuler 50 bp DNA ladder, **NK:** Negative Control

Table 14
Evaluation results of bands formed after screening with ORS 630 SSR primer by Logger Pro 3.16.2

Name of Genotype	9728-A	CL-217-R	IMI-044-A	3814-R
First Band	-	-	-	290 bp
Second Band	180 bp	180 bp	180 bp	180 bp

To observe the polymorphism formed as a result of bulk screening with ORS 630 in individuals and to determine how many of the individuals that formed the bulk gave similar results with their own bulk. Since 9728-A, CL-217-R, and IMI-044-A genotypes show a monomorphic band profile among themselves, it was considered sufficient to look at only one of the individuals forming the bulk. For this reason, only bulk individuals of IMI-044-A and 3814-R genotypes were screened. The screening result of the bulk and individuals of the IMI-044-A genotype was given in Figure 31, and the screening results of the bulk and individuals of the 3814-R genotype in Figure 32.

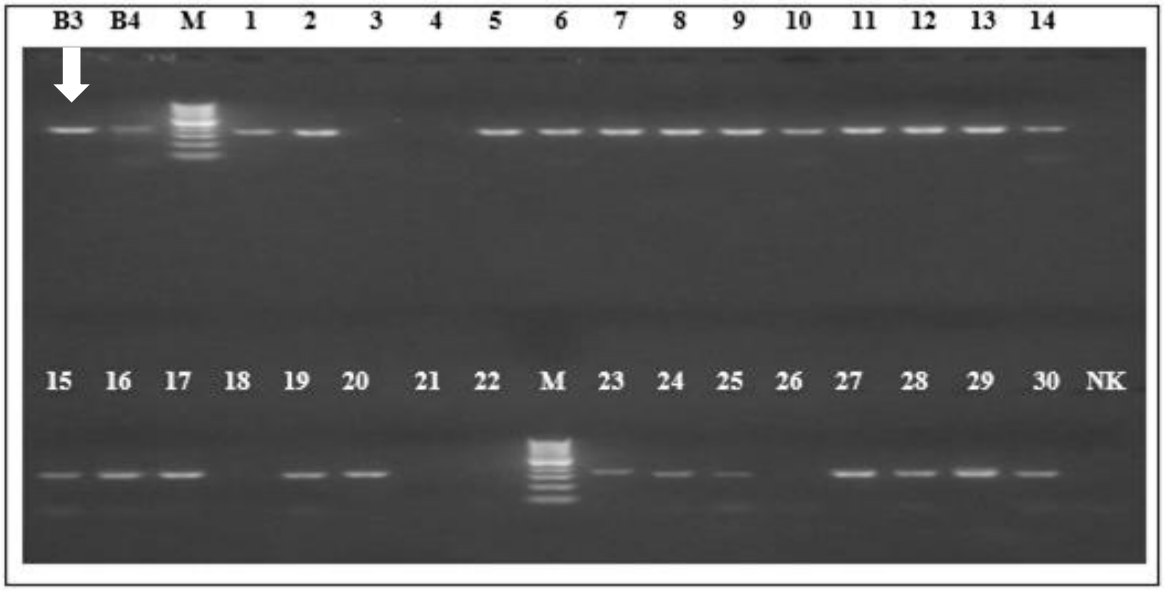


Figure 31. This image shows the PCR products of sunflower bulks and individuals of IMI-044-A genotypes obtained with ORS 630 SSR marker in 2% agarose gel.
B3: IMI-044-A bulk (ORS 630 marker), **B4:** 3814-R bulk (ORS 630 marker), **1-30:** Individuals of IMI-044-A genotypes (ORS 630 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

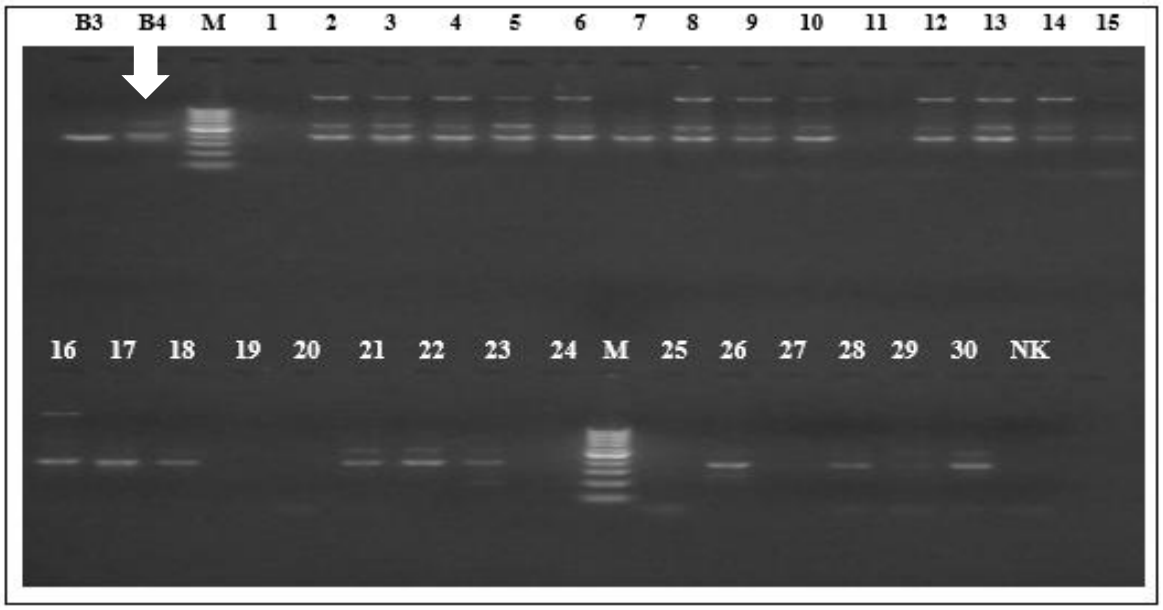


Figure 32. This image shows the PCR products of sunflower bulks and individuals of 3814-R genotypes obtained with ORS 630 SSR marker in 2% agarose gel.
B3: IMI-044-A bulk (ORS 630 marker), **B4:** 3814-R bulk (ORS 630 marker), **1-30:** Individuals of 3814-R genotypes (ORS 630 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

The screening results of F_2 individuals created using the IMI-044-A genotype and 3814-R genotypes were given in Figure 33.

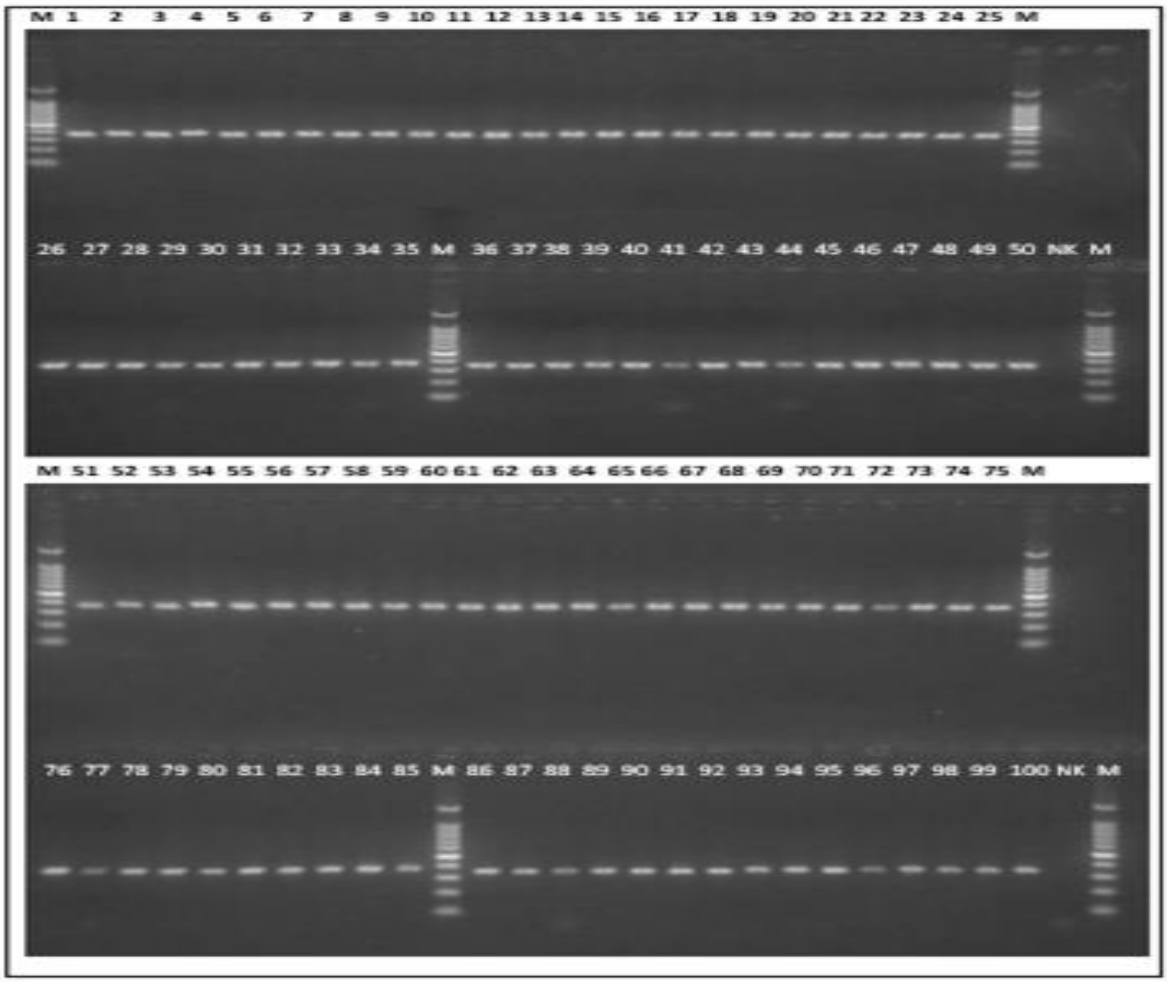


Figure 33. This image shows the PCR products of sunflower bulks and F_2 individuals formed by crossing IMI-044-A x 3814-R genotypes obtained with ORS 630 SSR marker in 2% agarose gel.
1-100: F_2 individuals (ORS 630 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

100 F_2 individuals were created using the IMI-044-A genotype, and 3814-R genotypes were screened with ORS 630 SSR primer. During the screening DNA pools, bands of two sequences of 180 bp and 290 bp lengths were observed in the 3814-R genotype, while a band of only 180 bp in length was observed in the 9728-A, CL-217-R, and IMI-044-A genotypes. When F_2 individuals were screened, only a 180 bp long PCR product band was observed in F_2 individuals created using the IMI-044-A and 3814-R genotypes.

4.3.5. Screening with ORS 928 SSR Primer

As a result of bulk screening with ORS 928 SSR primer, polymorphism between the formed band profiles was observed. DNA pool screening result was given in Figure 34. As a result of the screening, three bands were observed in the bulk of the 9728-A, CL-217-R, and IMI-044-A genotypes, while four band profiles were observed in the bulk screening of the 3814-R genotype. The lengths of the PCR products forming the bands are given in Table 15.

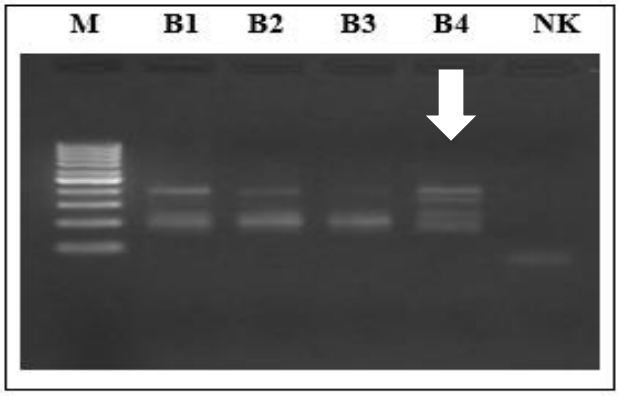


Figure 34. This image shows the PCR products of sunflower (*H. annuus* L.) genotypes bulks obtained with ORS 928 SSR marker in 2% agarose gel.
B1: 9728-A bulk (ORS 928 marker), **B2:** CL-217-R bulk (ORS 928 marker), **B3:** IMI-044-A bulk (ORS 928 marker), **B4:** 3814-R bulk (ORS 928 marker), **M:** Marker, GeneRuler 50 bp DNA ladder, **NK:** Negative Control

Table 15
Evaluation results of bands formed after screening with ORS 928 SSR primer by Logger Pro 3.16.2

Name of Genotype	9728-A	CL-217-R	IMI-044-A	3814-R
First Band	210 bp	210 bp	210 bp	210 bp
Second Band	-	-	-	175 bp
Third Band	130 bp	130 bp	130 bp	130 bp
Fourth Band	100 bp	100 bp	100 bp	100 bp

To observe the polymorphism formed as a result of bulk screening with ORS 928 in individuals and to determine how many of the individuals that formed the bulk gave similar results with their own bulk, ORS 928 screening of the individuals was also performed that formed bulk. Since 9728-A, CL-217-R, and IMI-044-A genotypes show a monomorphic band profile among themselves, it was considered sufficient to look at only one of the individuals forming the bulk. For this reason, only individuals of the bulk of IMI-044-A and 3814-R genotypes were screened. The screening results of the bulk and individuals of the IMI-044-A genotype were given in Figure 35, and the screening results of the bulk and individuals of the 3814-R genotype in Figure 36.

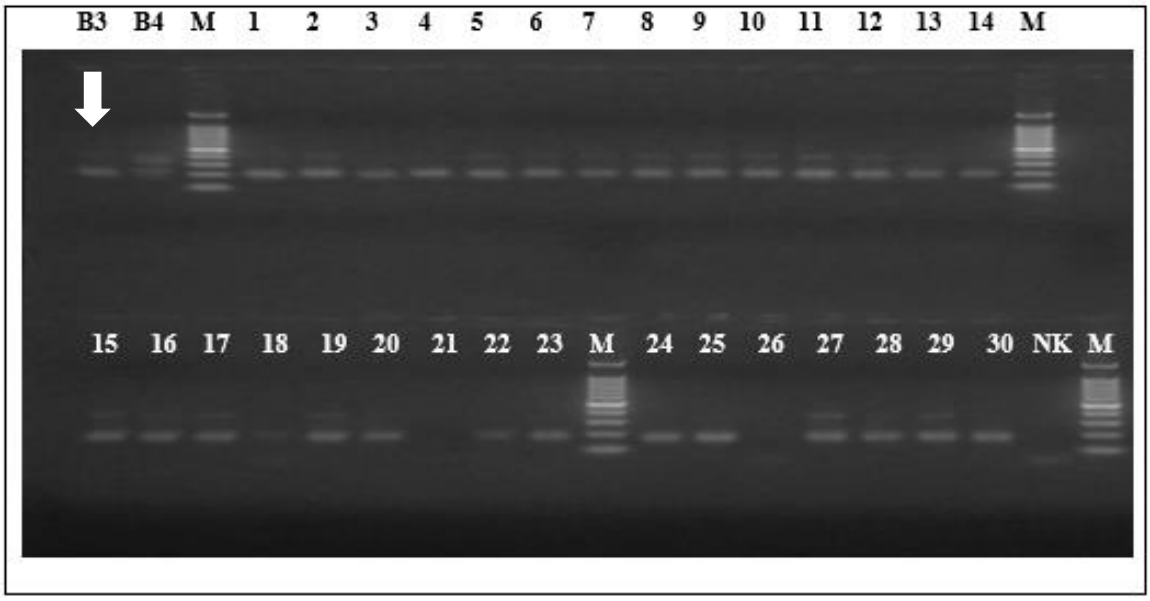


Figure 35. This image shows the PCR products of sunflower bulks and individuals of IMI-044-A genotypes obtained with ORS 928 SSR marker in 2% agarose gel.
B3: IMI-044-A bulk (ORS 928 marker), **B4:** 3814-R bulk (ORS 928 marker), **1-30:** Individuals of IMI-044-A genotypes (ORS 928 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

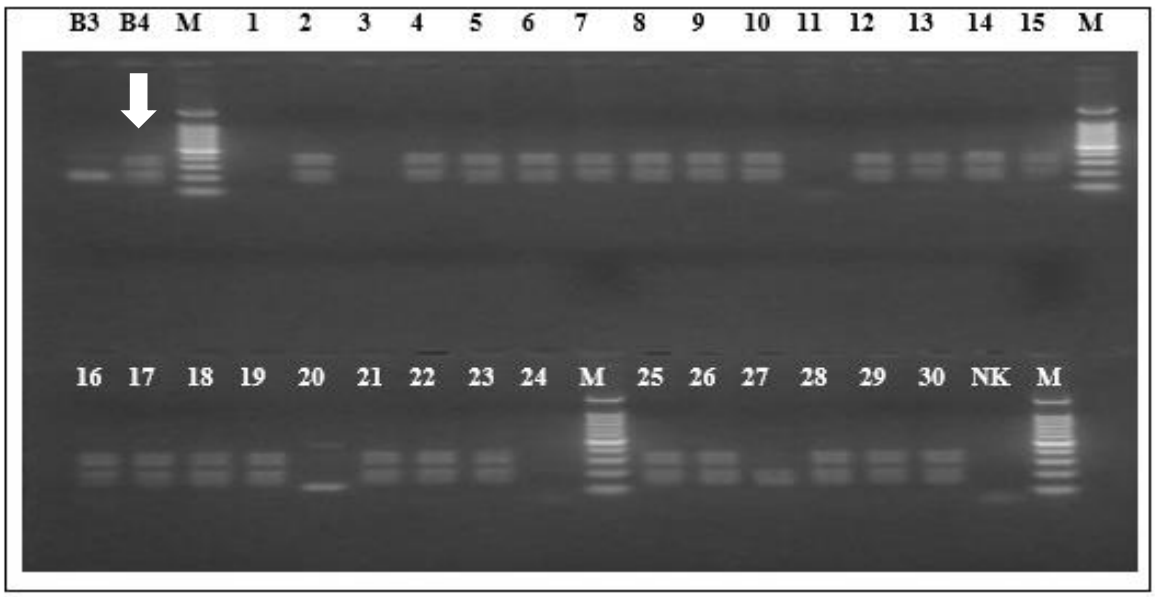


Figure 36. This image shows the PCR products of sunflower bulks and individuals of 3814-R genotypes obtained with ORS 928 SSR marker in 2% agarose gel.
B3: IMI-044-A bulk (ORS 928 marker), **B4:** 3814-R bulk (ORS 928 marker), **1-30:** Individuals of 3814-R genotypes (ORS 928 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

The screening results of F_2 individuals created using the IMI-044-A genotype and 3814-R genotypes were given in Figure 37.

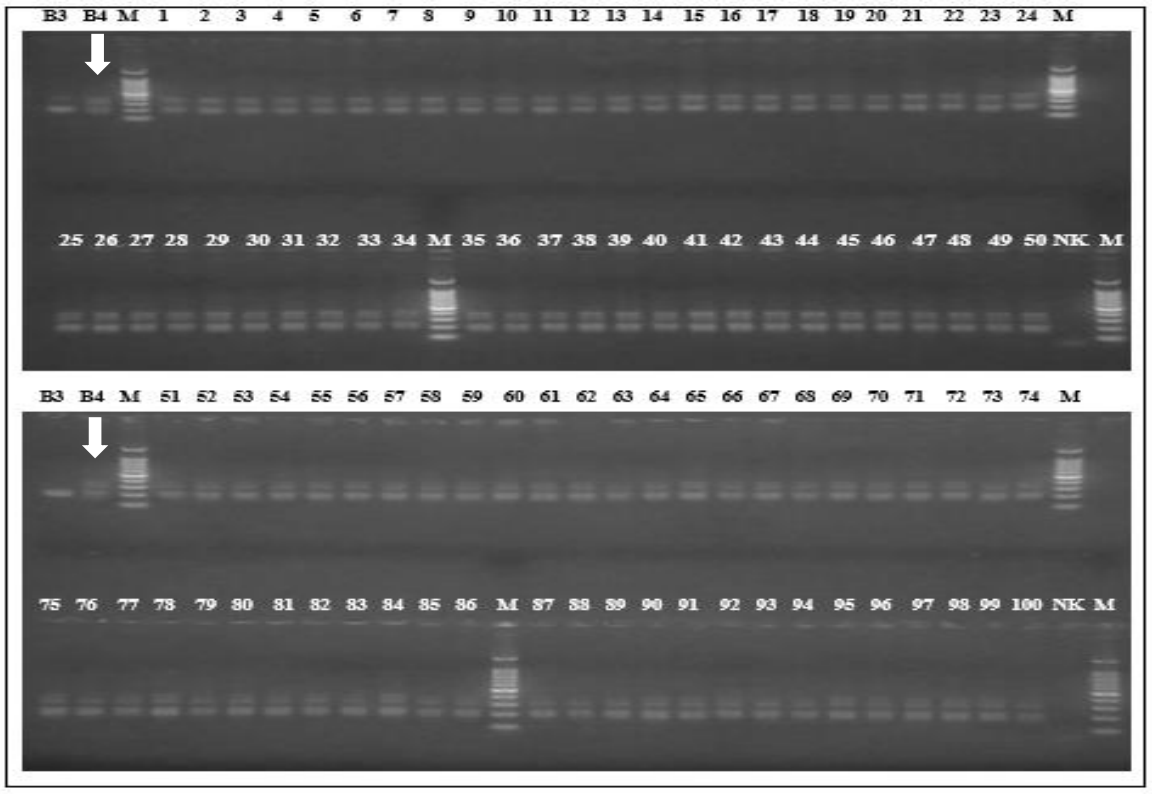


Figure 37. This image shows the PCR products of sunflower bulks and F_2 individuals formed by crossing IMI-044-A x 3814-R genotypes obtained with ORS 928 SSR marker in 2% agarose gel.

B3: IMI-044-A bulk (ORS 928 marker), **B4:** 3814-R bulk (ORS 928 marker), **1-100:** F_2 individuals (ORS 928 marker), **NK:** Negative Control, **M:** Marker, GeneRuler 50 bp DNA ladder

100 F_2 individuals were generated using the IMI-044-A genotype, and 3814-R genotypes were screened with ORS 928 SSR primer. During the screening of the bulks, bands of three sequences of 210 bp, 130 bp, and 100 bp lengths were observed in the 9728-A, CL-217-R, and IMI-044-A genotypes. Four different band profiles with a length of 210 bp, 175 bp, 130 bp, and 100 bp were observed in the 3814-R genotype. When the F_2 individuals were screened, three bands common to all genotypes were observed in F_2 individuals created using the IMI-044-A and 3814-R genotypes. A 175 bp long band was found only in the 3814-R genotype observed in F_2 individuals.

CHAPTER 5
RESULTS AND RECOMMENDATIONS

The production of hybrids consists of a plant pollination control genetic system consisting of maternal lines carrying cytoplasmic male sterility (CMS) genes and paternal lines with fertility restoring (*Rf*) genes (Horn et al. 2003).

Marking fertility-restoring genes is essential for selecting carriers of dominant and recessive alleles of *Rf* genes among segregated populations. This significantly speeds up the acquisition of parental hybrid forms. Therefore, searches of markers tightly associated with *Rf* genes are conducted worldwide; for example, several markers linked to the *Rf* gene have been identified in maize (Zhang et al., 2006), sunflower (Horn et al., 2003), soybean (Dong et al., 2012), barley (Ui et al., 2015).

In this study, instead of acquiring new molecular markers tightly linked to the *Rf* genes in sunflower, a screening study of sunflower genotypes that have commercial importance in Türkiye was performed. For this purpose, previously identified molecular markers tightly linked to the *Rf* genes were selected and used in screening studies.

In this thesis, four parental genotypes were analyzed. These were 9728-A and IMI-044-A genotypes that didn't carry the Rf gene, and CL-217-R and 3814-R genotypes that carried the *Rf* gene and F2 individuals were obtained by Thrace Agricultural Research Institute.

BSA analysis was performed with 21 SSR markers to parental individual pools. After five polymorphic primers were found, the screening of our parental individuals forming the bulk and our F2 individuals was performed.

As a result of BSA screening with the ORS 995 SSR primer, a band with a length of 135bp in both of the male sterile genotypes and a band with a length of 150 bp in two of the genotypes that were known to carry *Rf* gene, were detected. The same bands were found when the individuals of the genotypes that make up the bulk were screened. In the screening of F2 individuals performed later, only 150 bp long bands formed in individuals from both F2 generations were observed. For this reason, after screening results, ORS 995 SSR primer was thought will be helpful for hybrid seed production studies of commercial sunflower genotypes in Türkiye.

As a result of BSA screening with the ORS 511 SSR primer, band formation was not observed in the male sterile genotypes, while a band was detected with a length of 180 bp in two of the genotypes known to carry the *Rf* gene. The same bands were found when the individuals of the genotypes that make up the bulk were screened. In the subsequent scans of F_2 individuals, the same band with a length of 180 bp in both F_2 generations was observed. For this reason, after screening results, ORS 511 SSR primer was thought to be useful for hybrid seed production studies of commercial sunflower genotypes in Türkiye.

As a result of BSA screening with ORS 822 SSR primer, two types of bands, 170 bp and 80 bp in length, in the 9728-A, CL-217-R, and IMI-044-A genotypes were observed, but in the 3814-R genotype, a single band of 80 bp in length was observed. Because 9728-A, CL-217-R, and IMI-044-A genotypes were given the same bands, only individuals of IMI-044-A and 3814-R genotypes were screened and bands with the same lengths as their DNA pools were observed. In the screening of F_2 individuals that were created using the IMI-044-A and 3814-R genotypes, only a single band with a length of 80 bp was observed. Although the ORS 822 SSR primer was polymorphic for the 3814-R genotype, the F_2 generation result was not selective. For this reason, further studies will require the ORS 822 SSR primer to be used in future studies.

As a result of BSA screening with ORS 630 SSR primer, a single band with a length of 180 bp in the 9728-A, CL-217-R, and IMI-044-A genotypes was observed, and two types of bands with a length of 180 bp and 290 bp in the 3814-R genotype were observed. Because 9728-A, CL-217-R, and IMI-044-A genotypes were given the same bands, only individuals of IMI-044-A and 3814-R genotypes were screened and bands with the same lengths as their DNA pools were observed. In the screening of F_2 individuals that were created using the IMI-044-A and 3814-R genotypes, only a single band with a length of 180 bp was observed. Although the ORS 630 SSR primer was polymorphic for the 3814-R genotype, the F_2 generation result was not selective. For this reason, further studies will require for the ORS 630 SSR primer to be used in future studies.

As a result of BSA scanning with ORS 928 SSR primer, three types of bands with lengths of 210 bp, 130 bp, and 100 bp were observed in the 9728-A, CL-217-R, and IMI-044-A genotypes, but in the 3814-R genotype, four types of bands with a length of 210 bp, 175 bp, 130 bp, and 100 bp were observed. Since 9728-A, CL-217-R, and IMI-044-A genotypes were given the same bands, only individuals of IMI-044-A and 3814-R genotypes

were screened and bands with the same lengths with their DNA pools were observed. In the screening of F_2 individuals that were created using the IMI-044-A and 3814-R genotypes, the band that was specific to 3814-R with a length of 175 bp was also found. For this reason, after screening results, ORS 928 SSR primer was thought it will be useful for hybrid seed production studies of commercial sunflower genotypes in Türkiye.

REFERENCE

Abratti, G., Bazzalo, M. E., León, A. (2008). “Mapping a novel fertility restoration gene in sunflower”. In Proceedings of 17th International Sunflower Conference, Córdoba, Spain. 617-621.

Altinkut, A., Gozukirmizi, N. (2003). “Search for microsatellite markers associated with water-stress tolerance in wheat through bulked segregant analysis”. Molecular Biotechnology, 23 (2), 97-106.

Anisimova, I. N., Gavrilova, V. A., Rozhkova, V. T. (2009). “Molecular markers in identification of sunflower pollen fertility restorer genes”. Russ. Agricult. Sci. 35, 367–370.

Arif, I. A., Bakir, M. A., Khan, H. A., Al Farhan, A. H., Al Homaidan, A. A., Bahkali, A. H., Shobrak, M. (2010). “A brief review of molecular techniques to assess plant diversity”. International Journal of Molecular Sciences, 11 (5), 2079-2096.

Berry, S. T., Leon, A. J., Hanfrey, C. C., Challis, P., Burkholz, A., Barnes, S. R., Caligari, P. D. S. (1995). “Molecular marker analysis of Helianthus annuus L. 2. Construction of an RFLP linkage map for cultivated sunflower”. Theoretical and Applied Genetics, 91 (2), 195-199.

Bohra, A., Jha, U. C., Adhimoolam, P., Bisht, D., Singh, N. P. (2016). “Cytoplasmic male sterility (CMS) in hybrid breeding in field crops”. Plant Cell Reports, 35 (5), 967-993.

Dimitrijevic, A., Horn, R. (2018).” Sunflower hybrid breeding: from markers to genomic selection”. Frontiers in Plant Science, 8, 2238. https://doi.org/10.3389/fpls.2017.02238

Doyle, J.J., Doyle, J.L. (1987). “Isolation of DNA from fresh plant tissue”. Focus 12, 13-15.

Feng, J., Jan, C. C. (2008). “Introgression and molecular tagging of Rf 4, a new male fertility restoration gene from wild sunflower Helianthus maximiliani L”. Theoretical and Applied Genetics, 117 (2), 241-249.

Fernández-Martínez, J. M., Domínguez, J., Pérez-Vich, B., Velasco, L. (2010). “Update on breedıng for resistance to sunflower broomrape/actualızacıón de la sıtuacıón de la mejora genétıca de gırasol para resıstencıa al jopo”. Helia, 33 (52), 1-12. https://doi.org/10.2298/hel1052001f

Fernie, A. R., Tadmor, Y., Zamir, D. (2006). “Natural genetic variation for improving crop quality”. Current Opinion in Plant Biology, 9 (2), 196-202. ISSN 1369-5266, https://doi.org/10.1016/j.pbi.2006.01.010

Fick, G. N., Miller, J. F. (1997). “Sunflower breeding”. Sunflower Technology and Production, 35, 395-439.

Gentzbittel, L., Mestries, E., Mouzeyar, S., Mazeyrat, F., Badaoui, S., Vear, F., Nicolas, P. (1999). “A composite map of expressed sequences and phenotypic traits of the sunflower (Helianthus annuus L.) genome”. Theoretical and Applied Genetics, 99 (1), 218-234. https://doi.org/10.1007/s001220051228

Gentzbittel, L., Vear, F., Zhang, Y. X., Berville, A., Nicolas, P. (1995). “Development of a consensus linkage RFLP map of cultivated sunflower (Helianthus annuus L.)”. Theoretical and Applied Genetics, 90 (7), 1079-1086. https://doi.org/10.1007/BF00222925

Gupta, P. K., Varshney, R. K., Sharma, P. C., Ramesh, B. (1999). “Molecular markers and their applications in wheat breeding”. Plant Breeding, 118 (5), 369-390.

Horn, R., Kusterer, B., Lazarescu, E., Prüfe, M., Friedt, W. (2003). “Molecular mapping of the Rf1 gene restoring pollen fertility in PET1-based F1 hybrids in sunflower (Helianthus annuus L.)”. Theoretical and Applied Genetics, 106 (4), 599-606.

Hossam, M., I. (2012). “Response of some sunflower hybrids to different levels of plant density”. Apcbee Procedia, 4, 175-182.

Isaacs, S. M., Manivannan, N., Muralidharan, V. (2003). "Genetic diversity analysis using RAPD marker in inbred lines of sunflower (Helianthus annuus L.)". HELIA, 26 (39), 59-66.

Ivanov, M. K., Dymshits, G. M. (2007). “Cytoplasmic male sterility and restoration of pollen fertility in higher plants”. Russ. J. Genet. 43, 354–368.

Jan, C. C., Vick, B. A., Miller, J. F., Kahler, A. L., Butler III, E. T. (1998). "Construction of an RFLP linkage map for cultivated sunflower". Theoretical and Applied Genetics, 96 (1), 15-22.

Jarne, P., Lagoda, P. J. (1996). "Microsatellites, from molecules to populations and back". Trends in Ecology & Evolution, 11 (10), 424-429. ISSN 0169-5347. https://doi.org/10.1016/0169-5347(96)10049-5

Jocic, S., Miladinovic, D., Kaya, Y. (2015). "Breeding and genetics of sunflower. In Sunflower". 1-25. AOCS Press. https://doi.org/10.1016/B978-1-893997-94-3.50007-6

Jones, C. J., Edwards, K. J., Castaglione, S., Winfield, M. O., Sala, F., Van de Wiel, C., Karp, A. (1997). "Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories". Molecular Breeding, 3 (5), 381-390. https://doi.org/10.1023/A:1009612517139

Kaul, M. L. (1988). Malvaceae. In Male Sterility in Higher Plants, 705-716. Springer, Berlin, Heidelberg.

Kaya, Y., Evci, G., Durak, S., Pekcan, V., Gucer, T. (2009). "Yield components affecting seed yield and their relationships in sunflower (Helianthus annuus L.)". Pak. J. Bot, 41 (5), 2261-2269.

Kinman, M. L. (1970). "New developments in the USDA and state experiment station sunflower breeding programs". In Proceedings of the 4th International Sunflower Conference, 181-183. Paris: International Sunflower Association.

Konyali, S. (2017). "Sunflower production and agricultural policies in Turkey". Sosyal Bilimler Araştırma Dergisi, 6 (4), 11-19. ISSN: 214775237.

Kusterer, B., Horn, R., Friedt, W. (2005). "Molecular mapping of the fertility restoration locus Rf1 in sunflower and development of diagnostic markers for the restorer gene". Euphytica, 143 (1), 35-42.

Laser, K. D., Lersten, N. R. (1972). "Anatomy and cytology of microsporogenesis in cytoplasmic male sterile angiosperms". The Botanical Review, 38 (3), 425-454. https://doi.org/10.1007/BF02860010

Leclercq, P. (1969). “Une sterilite male cytoplasmique chez le tournesol”. Ann. Amel. Plantes, 19, 99-106.

Liu, X., Guo, L., You, J., Liu, X., He, Y., Yuan, J., Feng, Z. (2010). “Progress of segregation distortion in genetic mapping of plants”. Res J Agron, 4 (4), 78-83.

Liu, Z., Mulpuri, S., Feng, J., Vick, B. A., Jan, C. C. (2012). “Molecular mapping of the Rf 3 fertility restoration gene to facilitate its utilization in breeding confection sunflower”. Molecular Breeding, 29 (2), 275-284.

Liu, Z., Wang, D., Feng, J., Seiler, G. J., Cai, X., Jan, C. C. (2013). “Diversifying sunflower germplasm by integration and mapping of a novel male fertility restoration gene”. Genetics, 193 (3), 727-737. https://doi.org/10.1534/genetics.112.146092

Lu, Y. H., Melero-Vara, J. M., García-Tejada, J. A., Blanchard, P. (2000). “Development of SCAR markers linked to the gene Or5 conferring resistance to broomrape (Orobanche cumana Wallr.) in sunflower”. Theoretical and Applied Genetics, 100 (3), 625-632.

Moxon, E. R., Wills, C. (1999). “DNA microsatellites: agents of evolution?”. Scientific American, 280 (1), 94-99.

Mullis, K. B. (1990). “The unusual origin of the polymerase chain reaction”. Scientific American, 262 (4), 56-65.

Nimmakayala, P., Tomason, Y. R., Jeong, J., Vajja, G., Levi, A., Gibson, P., Reddy, U. K. (2009). “Molecular diversity in the Ukrainian melon collection as revealed by AFLPs and microsatellites”. Plant Genetic Resources, 7 (2), 127-134. doi:10.1017/S1479262108098481

Qi, L. L., Seiler, G. J., Vick, B. A., Gulya, T. J. (2012). “Genetics and mapping of the R 11 gene conferring resistance to recently emerged rust races, tightly linked to male fertility restoration, in sunflower (Helianthus annuus L.)”. Theoretical and Applied Genetics, 5 (125), 921-932.

Quagliaro, G., Vischi, M., Tyrka, M., Olivieri, A. M. (2001). “Identification of wild and cultivated sunflower for breeding purposes by AFLP markers”. Journal of Heredity, 92 (1), 38-42.

Sajer, O., Schirmak, U., Hamrit, S., & Horn, R. (2020). Mapping of the new fertility restorer gene Rf-PET2 close to Rf1 on linkage group 13 in sunflower (Helianthus annuus L.). Genes, 11 (3), 269.

Santalla, M., Power, J. B., Davey, M. R. (1998). “Genetic diversity in mung bean germplasm revealed by RAPD markers”. Plant Breeding, 117 (5), 473-478.

Schilling, E. E., Heiser, C. B. (1981). “Infrageneric classification of Helianthus (Compositae)”. Taxon, 30 (2), 393-403. https://doi.org/10.2307/1220139

Schnabel, U., Engelmann, U., Horn, R. (2008). “Development of markers for the use of the PEF1 cytoplasm in sunflower hybrid breeding”. Plant Breeding, 127 (6), 587-591. https://doi.org/10.1111/j.1439-0523.2008.01516.x

Schnable, P. S., Wise, R. P. (1998). “The molecular basis of cytoplasmic male sterility and fertility restoration”. Trends in Plant Science, 3 (5), 175-180. https://doi.org/10.1016/S1360-1385(98)01235-7

Semagn, K., Bjørnstad, Å., Ndjiondjop, M. N. (2006). “An overview of molecular marker methods for plants”. African Journal of Biotechnology, 5 (25), 2540-2568. ISSN 1684–5315.

Serieys, H. (1996). “Identification, study and utilization in breeding programs of new CMS sources”. Helia, 19 (special issue), 144-157.

Serieys, H. (2005). “Identification, study and utilization in breeding programs of new CMS sources, in the FAO Subnetwork”. In: Proceedings of 2005 sunflower subnetwork progress report, 17 (20), 47–53.

Talukder, Z. I., Ma, G., Hulke, B. S., Jan, C. C., Qi, L. (2019). “Linkage mapping and genome-wide association studies of the Rf gene cluster in sunflower (Helianthus annuus L.) and their distribution in world sunflower collections”. Frontiers in Genetics, 10, 216.

Tang, S. J. Y. K., Yu, J. K., Slabaugh, M. B., Shintani, D. K., Knapp, S. J. (2002). “Simple sequence repeat map of the sunflower genome”. Theoretical and Applied Genetics, 105 (8), 1124-1136.

Vear, F. F. (2016). “Changes in sunflower breeding over the last fifty years”. OCL Oilseeds and Fats Crops and Lipids, 23 (2), 1-8.

Vranceanu, A. V., Stoenescu, F. M. (1978). “Genes for pollen fertility restoration in sunflowers. Euphytica”. 27 (2), 617-627. https://doi.org/10.1007/BF00043193

Wenzl, P., Carling, J., Kudrna, D., Jaccoud, D., Huttner, E., Kleinhofs, A., & Kilian, A. (2004). “Diversity Arrays Technology (DArT) for whole-genome profiling of barley”. Proceedings of the National Academy of Sciences, 101 (26), 9915-9920.

Wieckhorst, S. (2012). Characterization of the Pl ARG locus mediating resistance against Plasmopara halstedii in sunflower (Doctoral dissertation, Technische Universität München).

Yu, J. K., Tang, S., Slabaugh, M. B., Heesacker, A., Cole, G., Herring, M., Knapp, S. J. (2003). “Towards a saturated molecular genetic linkage map for cultivated sunflower”. Crop Science, 43 (1), 367-387.

Yue, B. I. N. G., Vick, B. A., Cai, X. I. W. E. N., Hu, J. (2010). “Genetic mapping for the Rf1 (fertility restoration) gene in sunflower (Helianthus annuus L.) by SSR and TRAP markers”. Plant Breeding, 129 (1), 24-28.

Electronical Resources

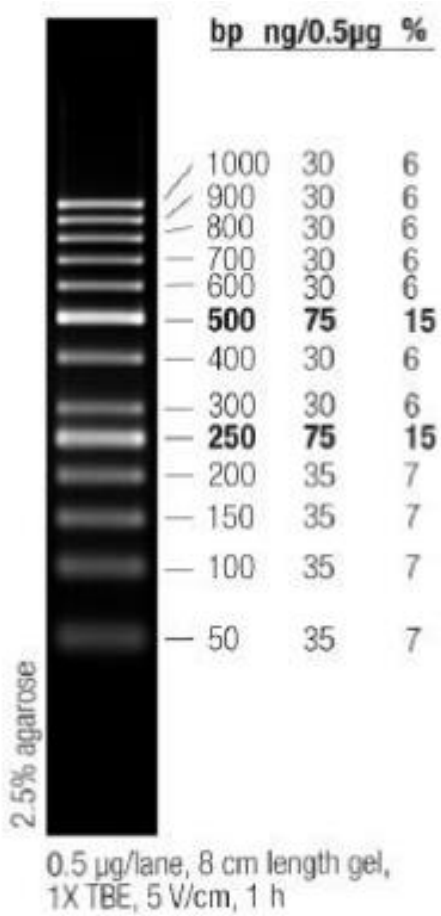
Food And Agriculture Organization Of The United Nations http://faostat.fao.org/site/339/default.aspx

Republic of Türkiye Ministry of Agriculture and Forestry, 2019

APPENDICES

APPENDIX 1

GENERULER 50 BP DNA LADDER



APPENDIX 2

LIST OF SSR PRIMERS

Name of SSR Primer	Forward Primer	Reverse Primer
ORS 13	gAATAACCTTgTggAgTTTgCC	CCTCATTCTCATTCTCTCCACC
ORS 45	AgAACgTATCTATCACgTgCCT	gATATTgAgCCTgACACTCACC
ORS 191	ACTgCgTTTgTgATTACTggTg	CATgCACTgAAgACATACACCC
ORS 224	AACCAAAgCgCTgAAgAAATC	TggACTAACTACCAgAAgCTAC
ORS 316	TggCgTCTTCATAgCATCAg	gAgATTTgAgCTTCgTgTTgC
ORS 317	TTTggCAgTTTggTggCTTA	GgTCgTATgCTTAATTCTTTCTCT
ORS 328	gACCTgTAggCCAATATgAgACTT	TTATACCggTgTTgTATCgTATCC
ORS 331	TgAAgAAgggTTgTTgATTACAAg	gCATTgggTTCACCATTTCT
ORS 488	CCCATTCACTCCTgTTTCCA	CTCCggTgAggATTTggATT
ORS 511	TggCTCAgATTAAgTTCACACAg	CgggTTgCgAgTAACAggTA
ORS 630	gCACgACCCggATATgTAAC	TgTgCTgAggATgATATgCAg
ORS 728	CTCCATAgCAACCACCTgAAA	CCAAACTCTgAATgATACTTgTgAC

ORS 799	ACTCCCTCCCATTCTCgTCT	TCCAgCAAgTCAgCAACAAC
ORS 822	CAATgCCATCTgTCATCAgCTAC	AAACAAACCTTTggACgAAACTC
ORS 849	AAgggCATCATAgTCAAACACCT	ACATCACCCACAACAACCATTAg
ORS 928	CATggTTATTTTggTTTgggTTT	gCTATTATCATgTCCTTgTCCTTTT
ORS 966	TCAAAgATgTCACCATAggAAAgA	ATTTgCTgAgACCATgAgCATC
ORS 995	CATgCTTTCTAggATggTCAgTT	TgTATgTggAggCCAACAAgTAT
ORS 1030	CCTTTgATgTAgTTAAggAAgTTgTg	CgATCAATTTATATgACCgAATTACC
ORS 1092	CCACgTCAgCATACCCAAATACT	gAgAACggTAAACAgTgAgAAAgg
ORS 1114	AgATggTggCAggAgAgTTAAAg	gCAgAAACAgATCAggAgggTAT