MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES
IN PURE AND APPLIED SCIENCES

USE OF KASPASSAY IN SEX DETERMINATION OF *PISTACIA*

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MASTER THESIS
Department of Bioengineering

Thesis Supervisor
Prof. Dr. Ahu ALTINKUT UNCUOĞLU

ISTANBUL, 2018

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ACKNOWLEDGEMENT

First of all; I would like to respectfully thank my hard-worker and successful supervisor Prof. Ahu ALTINKUT UNCUOĞLU and Assoc. Prof. Yıldız AYDIN for their efforts, guidance during my thesis study and for teaching me knowledge about science. They always show me how to maintain a well-disciplinated work.

I would also sincere thank all my lab colleagues Dr. Ezgi ÇABUK ŞAHİN, Nilay YÖNET, Elif KILIÇ and Çiğdem AYAN for all their support, favour and friendliness during the course of my thesis.

My deepest and special appreciation goes to my deary parrents Habibe & Ahmet Ümit ŞAHİN, my sister Elif Nur and my brothers Muhammed İrfan & Ömer Dündar. I'm very lucky that they are in my life with different colours. Especially I am grateful to my dearly mother for his patient, limitless love and effort to understand me. They are always starring characters of my cheering moments.

I would love to express a profound gratitude to my goody mentor Tatiana KAŞ for his sincere support every time I stumbled. Above all, I am feeling gratitude to my CREATOR for every piece of my life.

This research project has been supported by Marmara University Research Foundation (Project No: FEN-C-YLP-090217-0049) and dedicated to Turkish Armed Forces for their providing in a peace and security to us.

May, 2018 **Zeyneb Nur ŞAHİN**

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ABSTRACT

USE OF KASP ASSAY IN SEX DETERMINATION OF *PISTACIA*

The genus *Pistacia* is a perennial plant and belongs to Anacardiaceae (Cashew) family. The fruits of species have an extreme importance in food, health and baking industry as a raw material. While the species have different characters like membranous, leather, deciduous or evergreen, the most common known character is dioecism of individuals. The dioecious character (being a male/female) affects the anatomical or structural traits of individuals like picking season or fruit size so identify the individual is very crucial for producers. Marker Assisted Selection (MAS) is a promising process and may help to screen the sex or many other traits of plant seedlings quickly before they reach reproduction stage or show morphological data in more than several years. In this research, eight different *Pistacia* species were analyzed with Competitive Allele Specific PCR (KASP) assay technology as a marker screening system and it was focused on gender determination based on single nucleotide polymorphism (SNP). After the comprehensive literature search, 3 possible SNP were converted to KASP Primer according to their positions. On the result of KASP assay, 167992 (A/T) SNP discriminated the3female individuals of *Pistacia* which are *P. atlantica* Desf, *P. vera* L. and *P. terebinthus* L., 133396 (C/G) SNP primer discriminated only *P. vera* L. female and any polymorphism was not seen for SNP 176863 (A/G) position. These results were supportive to *Pistacia*'s ZW/ZZ (female/male) sex determination system because all discriminated females showed heterozygous character as reported. The data obtained from the study can directly influence the plant producers in terms of time and may be the predecessors of the upcoming studies about molecular sex markers. This research has been supported by Marmara University Research Foundation (Project No: FEN-C-YLP-090217-0049).

Keywords: KASP, *Pistacia*, SNP, sexual differentiation

ÖZET

PİSTACİA GENUS'UNDA CİNSİYET AYRIMININ KASP YÖNEMİYLE BELİRLENMESİ

Pistacia cinsi Anacardiaceae (Cashew) ailesine ait çok yıllık bir bitkidir. Türlere ait meyveler, gıda, sağlık ve fırıncılık endüstrisinde hammadde değeri itibariyle çok önemlidir. Türler membranöz veya deri yaprak, her mevsim yeşil veya kışın yaprak döken gibi farklı karakterlere sahipken, bilinen en yaygın genel özellikleri bireylerinin çift evcikli (dioik) oluşudur. Dioik karakter (erkek / dişi) bireylerin anatomik veya yapısal özelliklerini etkilemektedir; örneğin meyve toplanma mevsimi veya meyve boyutu bireyin erkek veya dişi oluşuna göre aynı türde değişiklik gösterir, bu durum ise üreticiler için çok önemlidir. Markör destekli seleksiyon (MAS) umut verici bir yöntemdir. Cinsiyetin veya diğer birçok karakterin belirlenmesinde bitkilerin çoğalma aşamasına gelmesini ve biçimsel özellikler gösteresini beklemeden kısa süre içerisinde kesin sonuçlar almamıza yardımcı olur. Bu araştırmada, sekiz farklı *Pistacia* türüiçinKASP (Kompetitif Allel Spesifik PZR)analizteknolojisikullanılmış ve tek nükleotid polimorfizmine (SNP) dayalı olasıcinsiyetler markörlerine odaklanılmıştır. Kapsamlı bir literatür taramasından sonra 3 muhtemel SNP, KASP primer çiftlerine dönüştürüldü. KASP analizleri sonucunda, 167992 (A/T) SNP primerinin *P. atlantica, P. vera* ve *P. terebinthus* türlerinin dişi bireylerini, 133396 (C/G) SNP primerinin sadece P. vera dişi bireyini ayır ederken, 176863 (A/G) SNP primeri için herhangi bir polimorfizm görülmediğini gözlemlendi. Bu sonuçlar, *Pistacia*'nın ZW/ZZ (Dişi/Erkek) cinsiyet belirleme sistemini de tüm ayrım görülen dişi bireylerin heterezigot karakter göstermesi yönünden desteklemiştir. Çalışmadan elde edilen veriler, bitki üreticilerini zaman açısından doğrudan etkileyebileceği gibi ve moleküler cinsiyet belirteçlerinde gelecek çalışmalara öncül niteliğindedir. Bu araştırma Marmara Üniversitesi Bilimsel Araştırma Projeleri Birimi (Proje No: FEN-C-YLP-090217-0049) tarafından desteklenmiştir.

Anahtar Kelimeler: KASP, *Pistacia*, SNP, cinsiyet ayrımı

SYMBOLS

%	: Percentage
&	: And
°C	: Centigrade degrees
Δ	: Delta
Δ **Rn**	: Change in normalized reporter value
2n	: Diploid chromosome number
µl	: Mikroliter
bp	: base pair
cd	: Candela
cm	: Centimeter
k	: Kilo
m	: Meter
Mb	: Megabase
mg	: Miligram
min	: Minute
mL	: Milliliter
mm	: Milimeter
mM	: Milimolar
n	: Haploid chromosome number
rpm	: Revolution per Minute
Rn	: Normalised Reporter
s	: Second

ABBREVIATIONS

A : Adenine nucleotide

A/ T : Adenine or Thymine nucleotide

A/ G : Adenine or Guanine nucleotide

AFLP : Amplified Fragment Length Polymorphisms

APS : Allele Specific Primer

BC : Before Christ

bp : Base pair

BR : Broad-Range

C : Cytosine

C/G : Cytosine or Guanine nucleotide

CTAB : Cetyl trimethylammonium bromide

DNA : Deoxyribo Nucleic Acid

dsDNA : Double stranded DNA

EDTA : Ethylenediaminetetraacetic acid

FAM : 6-carboxyfluorescein

FAO : Food and Agriculture Organization of the United Nations

FRET : Resonance Energy Transfer

FTP : File Transfer Protocol

HEX : Hexachlorofluorescein

HRM : High Resolution Melting

ISSR : Inter Simple Sequence Repeat

KASP : Competitive Allele Specific PCR

NFQ-MGB : 3' nonfluorescent quencher- minor groove binder

MM : Molecular Marker

NAM	: Nested Association Mapping
NCBI	: National Center for Biotechnology Information
PBD	: Point Base Deletion
PCR	: Polymerase Chain Reaction,
PE	: Paired End
PVP	: PolyVinylPyrrolidone
QTL	: Quantitative Train Loci
RAPD	: Random Amplified Polymorphic DNA
RFLP	: Restriction Fragment Length Polymorphism
RNA	: Ribonucleic Acid
SCAR	: Sequence Characterized Amplified Region
SNP	: Single Nucleotide Polymorphism
SOAP	: Short Oligonucleotide Analysis Package
SRA	: Sequence Read Archive
SSD	: Sexual Size Dimorphism
SSR	: Simple Sequence Repeats
T	: Thymine
WGS	: Whole Genome Sequencing
ZW/ZZ	: Heterogametic female and homogametic male chromosomal sex-determination system

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1. INTRODUCTION

1.1. *Pistacia* Genus

The genus *Pistacia* is a perennial plant and belongs to Anacardiaceae (Cashew) family (Zohary, 1952). The genus consists more than 11 species (Kafkas, 2006). *Pistacia vera* L. is believed to be the most ancestral species on genus and the other species are probably its derivatives (Zohary, 1952). *Pistacia* is compatible plant with diverse climate condition and it's mostly distributed along Middle Eastern and Mediterranean basin shores (Kozhoride, 2015). The species may found as a form of tree or shrub but both forms is xerophytic (Al-Saghir, 2010).The first known classification study of the genus was performed by Zohary (1952). This classification was made using morphological character of plants. The latest taxonomic classification is supported by also molecular data of *Pistacia* genus and it is shown below (Amon, 2009; UniProt, 2017).

- Domain: Eukaryota
- Kingdom: Plantae
- Phylum: Tracheobionta
- Class: Magnoliopsida
- Subclass: Rosidae
- Order: Sapindales
- Family: Anacardiacae
- Genus: *Pistacia*
- Species: *Pistacia* sp.

It is thought that *Pistacia* came from Central Asia 80 million years ago (Parfitt and Banedess, 1997). Diversity centers are defined as Southern Europe, North Africa & Mediterranean region of the Middle East and Western & Central Asia (Kafkas and Perl-Treves, 2002; Kafkas et al., 2002). The species naturally travels from North Africa to the Philippines, from Texas to Honduras (AL-Saghir, 2010).

Species have different character of leaves like membranous, leather, deciduous, evergreen, stipule and trifoliate. Besides, the most common known characteristics of the

species was being dioecious, wind pollination (Anemophilous), homeochlamydic perianth (Mabberley, 1997) and have unisexual flowers. But only *Pistacia atlantica* species was identified as a monoecious exception (Kafkas et al., 2000; Isfendiyaroğlu & Özeker, 2009). One of the essential and common character for *Pistacia* genus is sex differences for individual. This Sexual Size Dimorphism of species (SSD) effects from fertilization season to individual body or fruit size (Fairbairn, 1997).

Figure 1.1. *Pistacia lentiscus* tree as an example of the genus

The current all studies showed that different chromosome numbers of same species have been detected. These differences might be caused by the small size of *Pistacia*'s chromosomes (Basr-ila et al., 2003). For example, while the chromosome number of *Pistacia eurycarpa* was reported as 2n = 28 (diploid) by Ghaffari and Harandi (2001), H. Basr-ila (2003) identified this number as 2n = 30.

Pistacia has an extensive usage platform from drug which use for reproduction dysfunction (Mosbah et al., 2016) to biodiesel (Samani et al., 2016). On the other hand, its importance comes from economic values, wild growing and climate tolerance (Al-Saghir and Porter, 2012).

The known *Pistacia* species are; *Pistacia vera* L. (Pistachio), *P. terebinthus* L., P. *lentiscus* L, *P. mutica* Fisch. & Mey., *P.integerrima* Stew. ex Brand., *P. atlantica* Desf, *P. palaestina*, *P. khinjuk* Stocks, *P. chinensi* Bunge, *P. eurycarpa* Yalt., *P. mexicana* Humb., *P. falcata* Rech., *P. weinmannifolia* Poiss., *P. texana* Swingle (Al-Saghir, 2006, 2010).

Pistacia vera (Pistachio)

Pistacia vera L. (pistachio) is the well-known species because of its edible fruit pistachio. It has increasing economic value on food, health and baking industry. Even it is referred to as the ‘‘green gold’’ due to this high economic value (Benmahioul 2012).

Pistachio mostly cultivated in Mediterranean region. The tree is found as female and male individuals which are shrub with frequent branches. The trees of species can be up to 10 m (meter) high. They have deciduous and large leaves (10.2-17.0 cm long, 8.4-16.0 cm wide); odd-pinnate, leathery and flattened petiole. It has small, multiplexed and clustered flowers. The seeds contain large cotyledons and its edible (Al-saghir, 2006).

The chromosome number of pistachio were decided 2n=30 like other 3 members of genus (*P. terebinthus* L., P. *atlantica* Desf. and P. eurycarpa Yalt.) (Basr-Ila et al., 2003). Although there are not so many genomic studies related genome size, the defined genome size is about 600 Mb and it is highly heterozygous (Motalebipour, 2016).

The leaf or nut extract of pistachio shows antibacterial, antifungal, antiviral (Özçelik et al., 2005) anti-inflammatory and antinociceptive (Orhan et al., 2006) activities and this gives new paths to the healthcare industry. Kernels of *P. vera* are remarkably rich in linoleic and linoleic acids, the fatty acids vital for human health (Garcia et al., 1992; Maskan and Karatas, 1998; Aslan et al., 2002; Satil et al., 2003).

Pistachio extract is often used as a flavour enhancer in desserts such as cakes, cookies or ice creams because the extract has a well pairing with vanilla and chocolate tastes. Primarily producers of *Pistacia vera* are respectively USA, Iran, Turkey and China.

While Turkey production *average* is almost 170,000 tonnes in 2016, USA's production is higher than 400 kilo tonnes so we have to spend more effort to increase production rate in worldwide (FAO, 2017).

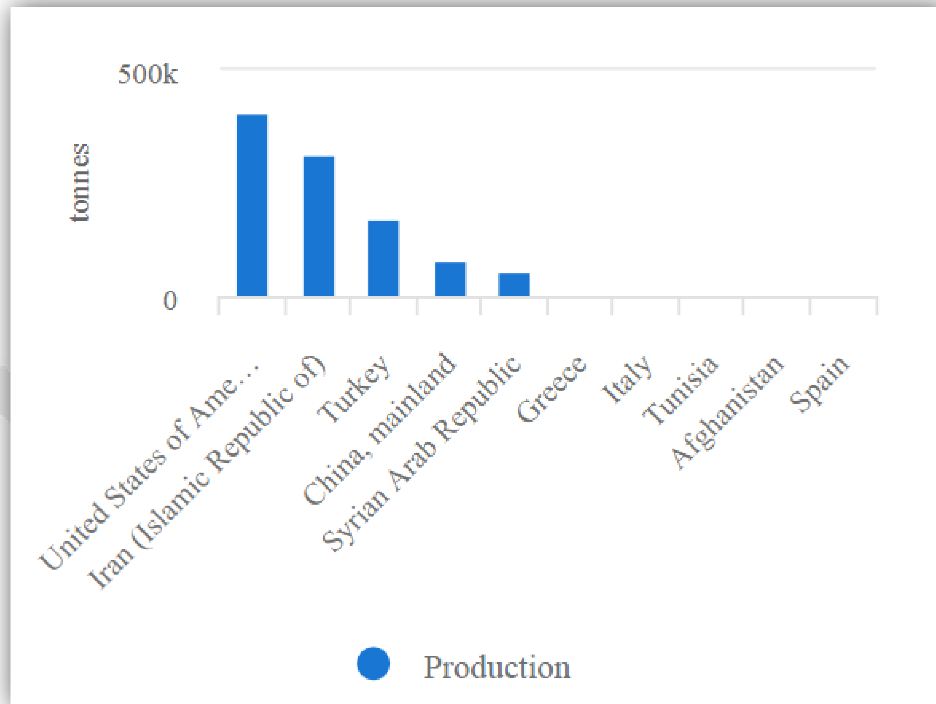

Figure 1.2. Production amount of Pistachios (*Pistacia vera*) in kilo tonnes: top 10 producers in 2016 (FAO, 2017).

The hull of pistachio seed does not use commonly as raw material like many other nuts and high amount of hulls become garbage. In Barreca's study (2016), the shells of mature pistachios were extracted with two organic solvents which are ethanol and methanol. Then they were characterized for cytoprotective activity, antioxidant power and phenolic composition. The results obtained highlight antioxidant and cytoprotective properties that are highly effective against a great variety of radical reactive species; these findings directly correlated to the high total phenol contents, in particular flavonols (Barreca et al., 2016).

Pistacia lentiscus

The common name of *Pistacia lentiscus* is "mastic tree" because of its useful resin; mastic. The mastic collection is done by scratching to bole or boughs and collection is

only made from male trees. Importance of *Pistacia lentiscus* plant comes from its mastic source. The chewing gum which is found mostly in the Chinos Island is known as "ScHINOS" in that region.

Mastic tree is a plant species in the form of shrub and these small trees (1-4 m high) are evergreen. That grows by itself in almost all Mediterranean shores. Leaves are small (2.0- 10.0 cm long, 3.4-10.0 cm wide), frequently, even-pinnate, leathery, glabrous, and elliptical form.

Since ancient times, the gum has been used for medical value. In the 1st century BC, Discorides and Scribonius Largus talked about the effects of gum resin on blood clotting, cough suppression, stomach burning and dental cleansing effects. The earliest source of the use of gum resins is reported by Herodotus in the 5th century BC. Heredotus described that the ancient Egyptian deaths were mummified with gum resin (Colombini et al., 2000).

In the Ottoman Empire period, the gum island maintained its importance. Residents had many different privileges as low taxes. At the present, the trade of chewing gum, commercial chewing gum and raw chewing gum is protected by the European Union since 1997 (Onay et al, 2016).

The chromosome number of *Pistacia lentiscus* L. is 2n=30 in both male & female species (Al-Saghir, 2014) and current genome size of species is 581 Mb (Siljak-Yakovlev, 2010).

Pistacia terebinthus (Menengiç)

Pistacia terebinthus L., known as terebinth or turpentine tree, is a perennial and fructiferous plant, like many members of genus *Pistacia*. The locally name of *Pistacia terebinthus* is “menengiç” that is produced a rich mixture of substances like resin, flavonoids, essential oils, organic acids, proteins, sugars, tannins and flavonoids (Pulaj et al., 2016).

Shrubs of the species have 2-3 m high. Leaves are deciduous, odd or even pinnate, membrananceous and large (10.0-19.0 cm long, 6.0-19.0 cm wide) (Al-Saghir, 2006). The fruits of genus are panicle and obovate sphere form. Their a*vera*ge length is 6.1

mm; width is 5.3 mm; geometric mean diameter is 5.43 mm and unit mass of fruit is 0.0565 g. (Aydın, C., & Özcan, M. (2002))

Turpentine which is product of *Pistacia terebinthus* is used as a traditional medicine in different countries like Iran or Kosovo (Pulaj et al., 2016). It has antioxidant, antimicrobial, anti-inflammatory and cytotoxic features thanks to flavoid and phenolic ingredients (Topçu et al., 2007). *Pistacia terebithus* has also disinfectant effect on bronchitis and can use as an air purifier on cure of asthma (Mohagheghzadeh et al., 2010). There are a number of other uses of this species which are good for stomachache (Cakilcioglu & Turkoglu, 2010), stomach ulcers (Polat et al., 2013), headache, hypotensive and prostate troubles (Agelet & Valles, 2003).

The chromosome sizes are small, so there was a complexity about the number. At the end of the all works diploid number is decided 30 in 2014 (Basr-Ila et al., 2003, Al-Saghir 2014). In addition to this known genome size is 660 Mb for *Pistacia terebinthus* (Motalebipour, 2016).

Pistacia mutica

Pistacia mutica Fisch. & Mey has admitted as a subspecies of *Pistacia atlantica* (Rezaie, 2015). The tree (bene tree) is well-known with its fruit saqez in Iran as chewing gum. It has shown higher antioxidant activity than sesame and rice bran oils during frying of sunflower oil (Farhoosh et al., 2009).

Pistacia mutica is the most economically important species of Zagros forests in Iran because of it is compatibility with adverse environmental conditions such as semi-drought and sloping land (Pour et al., 2012). Tree of *Pistacia* can reach height of 2-7 m (Sabeti, 2006) and shows deciduous character. The a*vera*ges of leaves sizes are 11.5 cmlong and 9 cm wide and the mean of nut sizes are 6.2 x 8.0 x 5.2mm (length x width x thickness) (Karimi et al., 2009).

One of the most recent studies on this species is leaf biomass and leaf area analysing (Eslamdoust, 2017). According to this study *Pistacia* cultivators could decide water requirement or other requirement like environmental condition for plants.

The number of chromosomes in the *P. mutica* species is supposed to be the same as *Pistacia atlantica*, ie 2n = 30Basr-Ila et al., 2003, Al-Saghir, 2014), because of it is the

Pistacia atlantica subspecies. Also the genome size is 539 Mb (Preeze and Aradhya, 2016) for *Pistacia atlantica.*

There are also four more species which are used in this study which are *Pistacia integerrima, Pistacia atlantica, Pistacia khinjuk, Pistacia palaestina.Pistacia integerrima* which is also evergreen plant species accustom to high altitude conditions like 12,000 ft. (Uddin et. al., 2012). This species has ethanobotanically importance in traditional medical treatments of diseases which are chronic wounds, jaundice (Matin et. al., 2001), dysentery, coughs, phthisis (Bibi et al., 2011) and it also has central nervous system depressant activity (Uddin et. al., 2012). The chromosome number of *Pistacia integerrima* was defined 2n = 30 (Basr-Ila et al., 2003) and the genomic size of species has 588 Mb (Preeze and Aradhya, 2016). *Pistacia atlantica* trees have almost 7-meter-high and their leaves sizes are 8.0-17.6 cm long, 5.2-14.0 cm wide. Different parts of the species like fruit, leaves, resin or bark used for a wide range of purposes (Bozorgi et al., 2013) their some of the usage areas are respectively; eye infections (El-Hilaly et al., 2003), mouth disease (Altundag & Ozturk, 2011) and diabetes (Hamdan & Afifi, 2004). The trees of *Pistacia khinjuk* reported as, dimensionally different than other *Pistacia* species. The trees are small and fruit colours are blue and black. The altitude conditions are almost 2000 meter (Ahmed et al., 2017). The medical usages in this species are stomach discomfort, nausea, vomiting, motion sickness (Bozorgi et al., 2013) and use in veterinary (Bahmani & Eftekhari, 2013). *Pistacia palaestina* Boiss. (*Pistacia terebinthus* L. var *palaestina* (Boiss.) Engl.) is well-known with its fruit blended with other plants to make “zaater” as aromatic spice (Flamini et al., 2004). Like other species, *Pistacia palaestina* is a highly beneficial plant in the health field. Especially in Levant regions, it used as a laxative, stimulant and aphrodisiac (Oran& Al-Eisawi, 1998). Additionally, there are many study about anti-insect effects of the gall on *P. palaestina* (Martinez J.J.I, 2010; Rand et al., 2014; Flamini et al., 2004).

1.2. The Importance of Sex Determination on Plants

Sexual polymorphism is the anatomical and structural traits of male and female individuals. This difference affects many features of plants, especially gamete formation, size of leaf length and yield period. The determinations of sexual phenotype in plants are diverse; ranging from sex determination, hormonal regulation or cross-talk

between individuals (Tanurdzic and Banks, 2004). Rudolf Jakob Camerarius (1665-1721) is one of the pioneers in the plant sexuality. He had tried to understand the function of sexual parts (pistil and stamen) of flower and published his own experiments with “De Sexu Plantarum” at 1694 (Faegri et al., 2013)

Plants may sexually monomorphic (bisexual) or polymorphic (Charlesworth 2002) (**Figure 1.3**). A species is bisexual if the male and female flowers occur on the same plant. Bisexual plants may be hermaphroditic, when the two sexes are found in the same flower, or monoecious when they are restricted to separate flowers on the same plant. (Westergaard 1958). In sexually polymorphic plants species are unisexual or dioecious when the male (♂ staminate) and female (♀ pistillate) flowers occur on different individuals (Westergaard 1958).

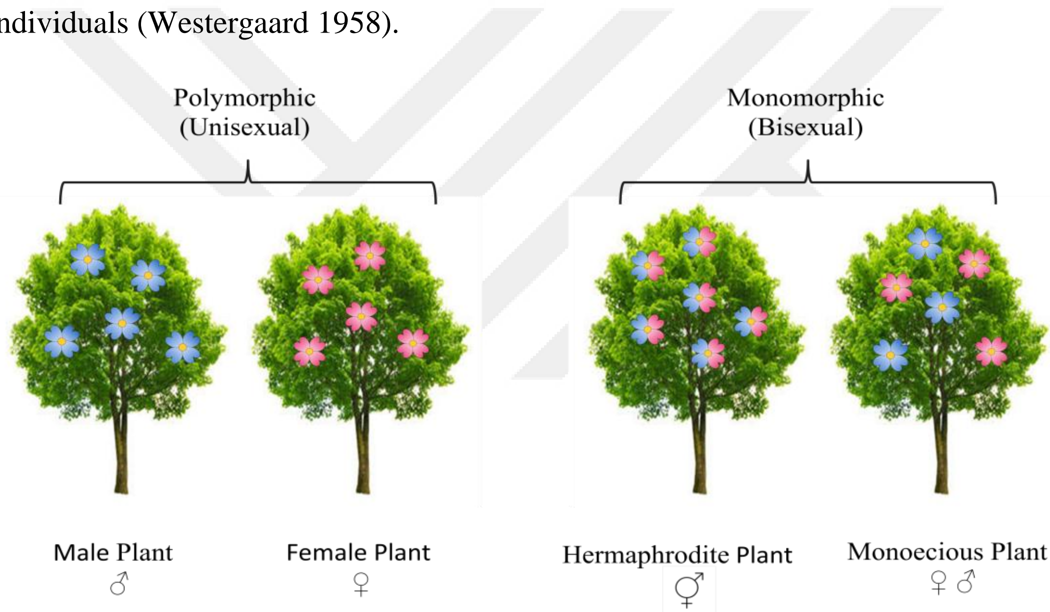

Figure 1.3. Sexuality in Flowering Plants

Many dioecious plants display sexual dimorphism in resource allocation across the life cycle. Females and males may differ in both the timing of allocation to various functions and in the absolute amount of resources allocated to above- and belowground vegetative growth and sexual reproduction (Putwain & Harper 1972; Delph 1999).

Sexual size dimorphic (SSD) fruit trees require a nearby tree of the opposite sex for fruit to be produced. The fruit is taken from female ones. It is unclear whether the tree is a tree or a female until about three years old in some plants. Male flowers usually have small or no stigmas in them; female ones will be missing stamens (Teitel et al., 2016). Among long-lived trees and woody shrubs, females tend to flower later and be smaller

than males at reproductive maturity (Obeso 1997; Nicotra 1999). For all these reasons, identified molecular marker may help to screen the sex of plant seedlings quickly before they reach reproduction stage.

In woody plant system, there is no need 1 male per 1 female. Generally, 1 male will serve to pollinate a number of female plants and exact range varies by type of plant. For instance, 1 male pistachio or male date palm can serve for 40 to 50 females. Sometimes, a branch from the opposite sex is even grafted onto a tree for ensuring that opposite flowers are found nearby to provide proper pollination (Oulton, 2004).

A particular approach to all plants would not unilaterally describe the complex sexual system; so, genetic, phylogenetic, epigenetic, cytogenetic studies will be necessary for future efforts (Harkess and Leebens-Mack, 2016). In this thesis we focused on determination of gender before maturing without the need for the formation of morphological data to earn time (between 5-8 year) (Hormaza et al., 1994).

1.3. Molecular Markers

Molecular markers are nucleic acid (DNA/RNA) fragments that are any gene region or associated with genes in the genome (Devran, 2003). These markers can be used for a variety of purposes ranging from creating of genetic and character maps of families to identifying and defining species (Rafalski, 2002). Mainly molecular markers (M.M.) can be categorized in 3 base; chip base M.M., hybridization base M.M. and polymerization base M.M's. While DNA amount have lesser importance on polymerization based techniques, qualified and quatified template DNA is required on hybridization based tecniques.

The main ones of the molecular markers are; RFLP, RAPD, AFLP, SSR and SNP (Staub et al., 1996). Each has its advantages and disadvantages. For example, too much DNA is needed for the Restriction Fragment Length Polymorphism (RFLP) method, which is also an expensive and time-consuming marker system. On the other hand, the results are highly reliable and genotypically heterozygous / homozygote discriminatory (codominant marker) (Ahn and Tanksley, 1993). RAPD (Random Amplified Polymorphic DNA) is based mainly on Polymerase Chain Reaction (PCR). Applying technique is simple and fast. There is also no need for sequence analysis to use this technique. However, only the formation of dominant markers and even minor changes

in the PCR effect on results are disadvantages of this technique (Williams et al., 1990). There are also comprehensive studies which used different molecular markers together on the same study to make sense of genetic diversity between species (Abuduli et al., 2016).

From this point of view, the marker system that thought is most advantageous in terms of repeatable and reliable is SNP (Single Nucleotide Polymorphism), which is the single nucleotide polymorphism marker. Since this type of marker is primarily a high-throughput marker (Mammadov et al., 2012), a single nucleotide sequence difference is the most common polymorphism in the genome, thus providing a more detailed study.

A Single Nucleotide Polymorphism (SNP) is called a single point mutation in the gene (or genome) sequence of an individual (**Figure 1.4**). This difference can occur in many species like plants, animals or bacteria. Mutation factors may be sequence deletion, sequence addition, translocation, substitution or other external factors like ultraviolet light. A single nucleotide polymorphism usually appears in the non-coding parts of the DNA, if it occurs in the encoded part, it affects the amino acid sequence to be produced in that region, but this ratio is very small, so usually gene product does not change. The two main advantages of SNP usage are their abundance (e.g. 1/36 bp [1 SNP per 36 base pair] for Arabidopsis, 1/21 bp for potato and 1/78 bp for barley) on genomes and availability of high throughput SNP genotyping technologies (Henry, 2008).

Over the last 30 years, the use and development of molecular markers have seen tremendous progress. Markers linked to the Single Nucleotide Polymorphism (SNP) in the system and methods become the most attractive marker system with the increase in the information in the molecular genetics and the accompanying complex databases (Mammadov et al., 2012). SNP markers used on many plants species and many platforms like QTL (Quantitative Trait Locus) analysis on rice (Yu et al., 2011), nested association-mapping (NAM) on maize for complex trait flowering time (Buckler et al., 2009) or founding Gene-Specific diagnostic marker against fungal pathogens like leaf rust, stripe rust, and powdery mildew diseases for maize (Lagudah et al., 2009). Single nucleotide differences obtained after sequencing, particularly where genetic resources are limited, have become a preferred method for DNA-based comparison of wild and processed species (Sharpe et al., 2013; Lee et al., 2014).

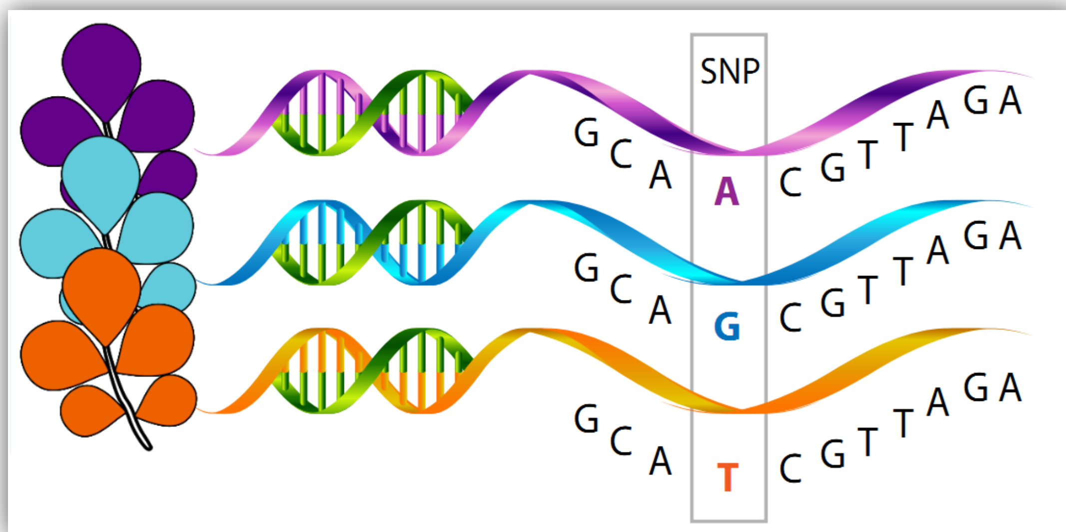

Figure 1.4. Illustration of Single Nucleotide Polymorphism

The generation of sex-linked markers is likely to reduce time, labour, and costs associated with breeding (Kafkas et al, 2015) or production-focused programs. For example, *Pistacia* species show their gender character after 5 to 8 year (Hormaza et al., 1994) without any molecular marker and female individuals' harvest, which means irrigation and maintains cost will continue for trees before yielding. In addition to that frequency of sex-linked markers depends of such factors as the chromosome number, the total size of the genome and the relative size of the gender-determining chromosomal segments (Esfendiyari et al., 2012).

1.4. Sex Discrimination in *Pistacia* Genus Based on Molecular Markers

The first sex determination study on *Pistacia* with molecular markers was done at 1994 by Hormaza. In this study 1,000 RAPD (Random Amplified Polymorphic DNA) markers had used for discrimination. Bulk *Pistacia vera* L. male and female samples had amplified with 700 decamer primers and as a result OPO08945 (OPO-08) had defined as a sex marker. 945 bp size band was observed on female bulk sample and this band was absent on male samples. In 2005 another study was reported to verify and develop OPO-08 RAPD primer with Touchdown PCR (Yakubov et al., 2005). Then researchers were determined PVF1-2 SCAR (Sequence Characterized Amplified Region) primers based on OPO-08. In that study 4-point base deletion (PBD) for female samples and 1 PBD for males were defined as a mutation.

In other completed gender discrimination study was associated RAPD markers in wild *Pistacia* which conducted under the leadership of Salih Kafkas (2001). BC156 (1300bp) & BC360 (500bp) primers for *Pistacia* eurocarpa and OPAK-09 (850bp) & BC346 (700bp) primers for *Pistacia atlantica*was indicated as a discrimination marker.

In the articles published by Esfendiyari (2010,2012), first 20 10-mer RAPD primersthan 30 different 10-mer RAPD primer were tried on 3 different species; *P. atlantica*, Desf subsp. *mutica*, P. *khinjuk* and P. *vera*. As a result, he reported that BC1200 primer is a discriminator primer and designed the primers PVF1 (forward) and PVF2 (reverse) from the PCR amplified region sequence (convert RAPD to SCAR). The SCAR primers which has 300 bp amplifying region, while generate a band in all female samples, there wasn't any band in male samples.

When studies are examined, it is seen that many of them are based on *Pistacia vera* genus (Hormaza et al., 1994; Yakubov, 2005; Ehsanpour, 2008; Ehsanpour and Arab 2010; Kamiad, 2014; Kafkas et al., 2015) due to the world wide trade income. On this basis, in 2008, *P. vera* L. cultivars were generated with ISSR (Inter Simple Sequence Repeat) primers and 2 of them were able to distinguish genders. These primers are (AC) 8GC and (AC) 8TA 10-mer repeats and band sizes are 2400 (Ehsanpour, 2008). Next year same research group ere experienced 32 arbitrary primers for RAPD analysis and mentioned FPK 106 and FPK 105 but there is no data related to primer sequence.

Up to the present, the most reliable study about sex linked marker on *Pistacia* genus is “Identification of sex-linked SNP markers using RAD sequencing suggests ZW/ZZ sex determination in *Pistacia vera* L.” (Kafkas et al., 2015). In that study 38 putative sex-associated SNP markers were identified as heterozygous in female individuals and homozygous in male individuals using RAD seq. suggests a ZW/ZZ sex determination system in pistachio. Most importantly, the determined SNP positions light on our Competitive Allele Specific PCR (KASP) system. 3 SNP were picked out among them and KASP primers were designed accordingly.

1.5. Competitive Allele Specific PCR (KASP)

High-throughput SNP genotyping can perform on Fixes-array, Flexible or GBS (Genotyping by Sequencing) technologies. Illumina Infinium iSelect HD (High-Definition) and Affymetrix Axiom are several platform of fixed array technology.

While fixed-array has been accomplishing great things on large number of samples because of the higher cost it's not always convenience to use it. As for that Flexible technologies like TaqMan®, Douglas Array Type or KASP™ is preferable on certain fields like Agriculture or Personal Medicine (Thomson, 2014).

KASP (Competitive Allele-Specific PCR) is the PCR-based fluorescently-labelled marker scanning system which developed by KBioscience. One marker can be run and scan on real-time by fluorescent plate readers. KASP genotyping assay is a novel, easily repeatable, wieldy, fast and economic allele-specific assay (**Figure 1.5**).

Figure 1.5. Properties of KASP assay

The technology based on KASP assay is FRET (fluorescence resonance energy transfer) cassettes combination with Polymerase Chain Reaction system. FRET cassettes originate from fluorescent tagged dyes FAM (6-carboxyfluorescein) and HEX (hexa-chloro-fluorescein) with specific oligo tails and their complementary quencher molecules to stop signal generation before matching (**Figure 1.6**). Two unlabelled APS (allele-specific primer) is the other key point of the reaction. Dye's sequences are the same with extension part of our primers one to one and quencher have complementary of these extensions. At the end of the PCR cycles, used primer/primers are defined through signal frequency.

On the experiment flow, after first cycle of PCR, extension parts of primers are doubling and sequence which is non complementary to template but complementary to FRET dye is forming (**Figure 1.6**). On the second cycle, fluorescent tagged dyes are releasing from quencher for coming together with amplified primer extension sequence

and fluorescent signal forms (**Figure 1.6**). Signals can be detected using real-time PCR instrument or plate reader.

The new Assay Search tools offered by the LGS group made it easy to get validated SNP assays for our interesting species. Relevant KASP Genotyping Assays can be found using a variety of criteria including SNP ID, chromosome location and gene name. Sequential submission is not required. This tool's including species are human, maize, rice, soybean, tomato and lentil from various parts of the world like UK or Canada (LGC Limited, 2017).

The KASP has a wide range of usage on different livings and it can be used easily for genotyping with cost advantage. Soybean (Yuan et al., 2014), grain amaranth (Maughan et al., 2013) ash tree (Sollars et al., 2017) and apple (Baumgartner et al., 2016) are some of the plants species which KASP assay has successfully applied for breeding or genetic diversity to the present.

One of the most recent studies using this novel technology is detection of G2019S mutation in *LRRK2* gene which is so crucial sporadic Parkinson disease. Researchers were explored that KASP assay is more reliable, time and cost effective genotyping assay for routine screening than the Taqman assay and Sanger sequencing (Landoulsi et al., 2017). In addition to this, according to Semagn's review in 2014, KASP is 12.3-46.1 % less expensive than GoldenGate or BeadXpress platforms.

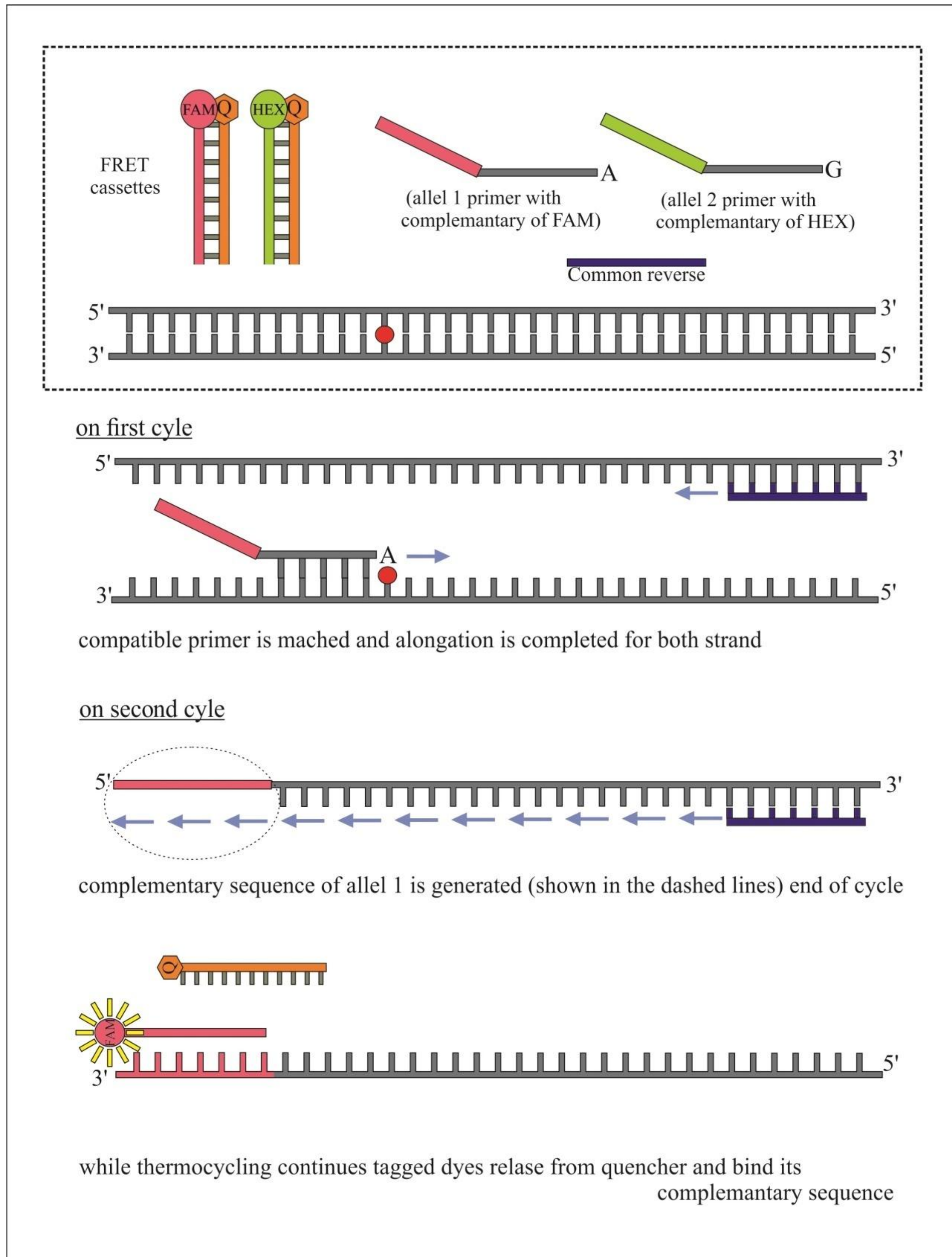

Figure 1.6. Signal Generation Flow Diagram on Thermocycling Process

2. MATERIAL and METHOD

The plan of study was designed as shown in the chart (**Figure 2.1**) and practiced step by step.

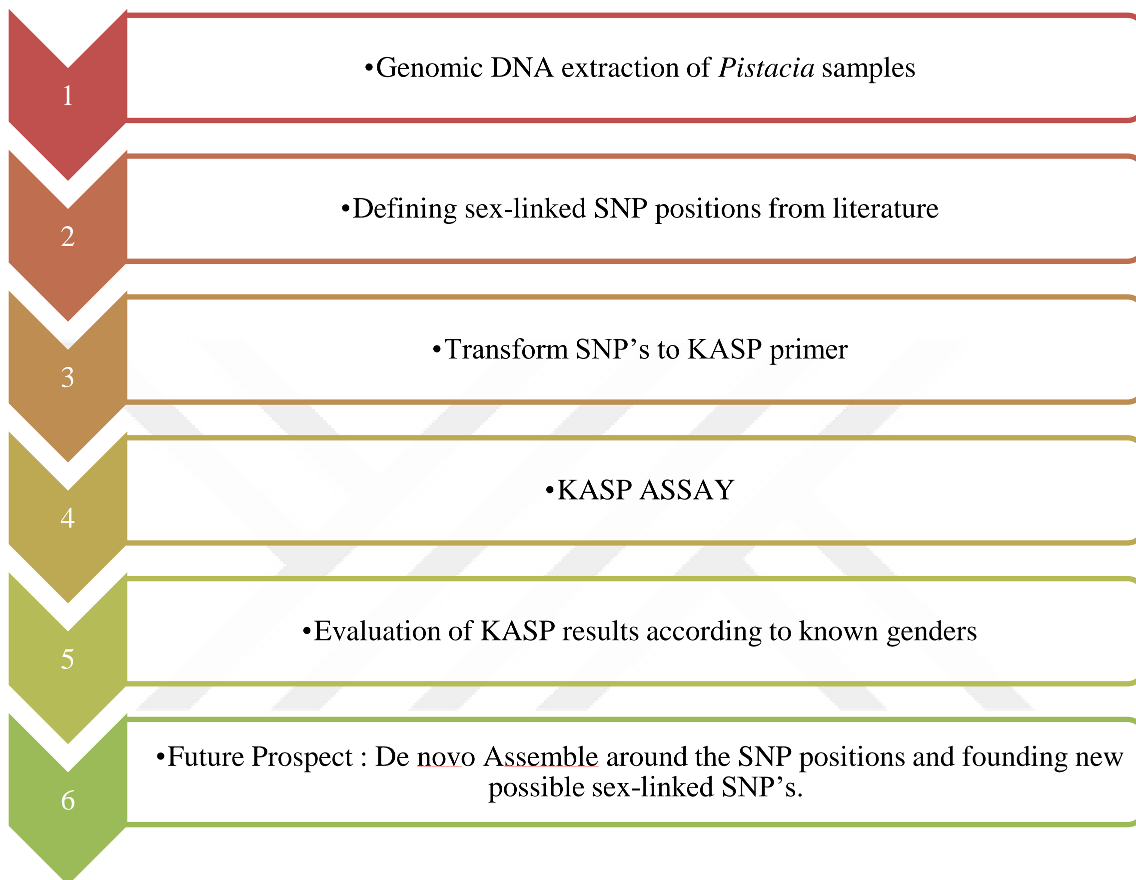

Figure 2.1. Working flow chart of thesis

2.1. Plant Material

As the plant material 7 males and 7 females (**Table 2.1**) *Pistacia* young leaf tissues were collected from Antepfıstığı Research Institute Collection Garden by Prof. Dr Ahmet Onay and in addition these 1 male and 1 female *Pistacia lentiscus* L. from Çeşme Çiftlikköy region in İzmir by the help of Metin Gemici for this study. Each sample leaf with tip part of branches was photographed in Marmara University Plant Biotechnology Laboratory (**Figure 2.2**).

Table 2.1. *Pistacia* samples (Sample number from 1 to 14 were came from Antepfıstığı Research Institute Collection Garden and 15th&16th samples were came from Çeşme Çiftlikköy region)

MALE SPECIES	FEMALE SPECIES
1. *Pistacia atlantica*	2. *Pistacia atlantica*
3. *Pistacia integerrima*	4. *Pistacia integerrima*
5. *Pistacia khinjuk*	6. *Pistacia khinjuk*
7. *Pistacia mutica*	8. *Pistacia mutica*
9. *Pistacia palaestina*	10. *Pistacia palaestina*
11. *Pistacia terebinthus*	12. *Pistacia terebinthus*
13. *Pistacia vera*	14. *Pistacia vera*
15. *Pistacia lentiscus*	16. *Pistacia lentiscus*

Collected fresh leaves were grinded for physical fractionation. For this purpose, each 16 individual's leaves were completely frozen and sterilized with liquid nitrogen then were grinded by Homogenizator &Metal beams until it became powder for 45 seconds at 25 frequencies. During these times it is very crucial that the samples remain frozen.

The powder samples were then stored at -80 ° C in the freezer on the Department of Biology at the Marmara University until DNA isolations were done.

2.2. Genomic DNA Extraction from *Pistacia* samples

Doyle and Doyle (1991) procedures were used for DNA isolation. This procedure was carried out with the following steps:

- Approximately 100 mg from each sample was weighted and transferred to 2 mL microtubes.
- For each sample, 600µl **CTAB** (Cetyl Trimethyl Ammonium Bromide) **solution** and 8mg **PVP** are mixed and heated to 60°C till dissolve.
- Than **0.2% β-Mercaptoethanol** was added into prepared solution.

- 600µlCTAB – PVP mixture was added into sample tubes and incubated at 60°C in Thermal Hot Block for 25min, tubes were shaken gently at each 5min.
- After that tubes were waited at room temperature for 5 – 10min.

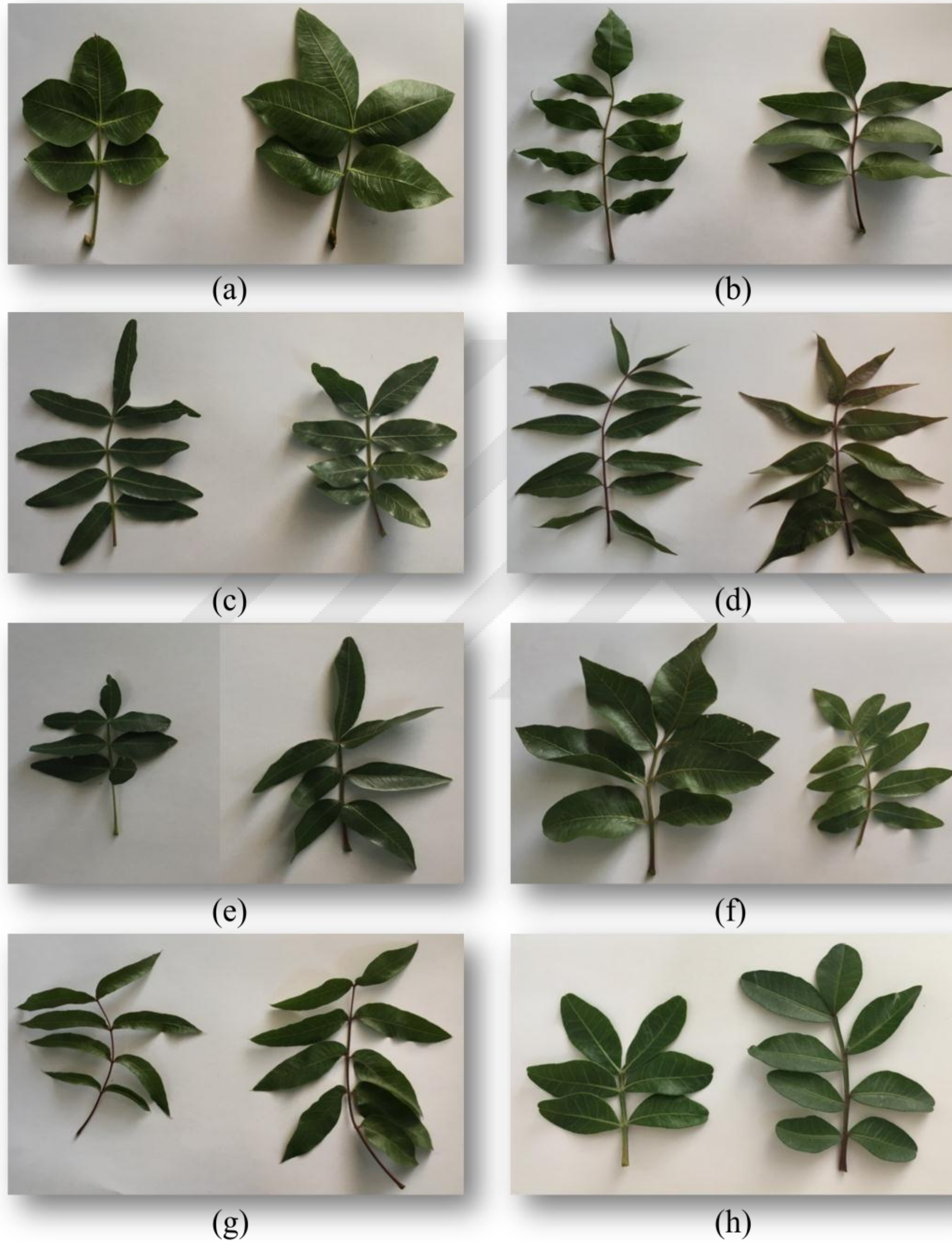

Figure 2.2. The male (left) and female (right) leaf samples for each species of *Pistacia* (a) *Pistacia vera* (b) *Pistacia terebinthus* (c) *Pistacia mutica* (d) *Pistacia integerrima* (e) *Pistacia atlantica* (f) *Pistacia khinjuk* (g) *Pistacia palaestina* (h) *Pistacia lentiscus.*

- 450µl**Chloroform:Octanol (24:1)** was added to each sample and inverted several time very slowly. (This step was done 2 times)
- Than samples were centrifuged at 13000rpm for 15min.
- Supernatants were transferred into new 2ml tubes by using slant micropipette tips.
- **NaCl** in ½ volume of supernatant and **95% ethanol** in 2 volumes of supernatant were added to samples. Samples were put into freezer (-20°C) for one night.
- After overnight incubation, samples were centrifuged at 13000rpm for 10min.
- Supernatants were removed. Adherent pellets to tube bottom were washed with 1000µL**75% ethanol** and 13000rpm centrifuge for 5min. (This step was repeated one more time)
- Supernatants were removed and pellets were dried by concentrator for10 minute.
- Pellets were dissolved in 100 µL **nuclease free water**.
- 2µL **RNase – A** is added and samples are incubated at 37°C for 30min.

DNAs were kept at -20°C for long term and +4°C for short term storage. Chemical Solutions and buffers used in this protocol are shown in the table below **(Table 2.2)**.

Table 2.2. Chemical solutions and buffers used in DNA isolation protocol.

Buffers	Components	Concantrations	Preperations
Extraction Buffer (CTAB)	Sodium EDTA (pH: 8.0) Tris-HCl NaCl CTAB	20 mM 100mM 1.4 M 2.0 % (w/v)	- 5g CTAB was put on bottle and dissolve in ~ 30 ml d-H_2O - Respectively 25mL 1M Tris-HCl, 70mL 5M NaCl and 10mL 0.5M EDTA were added and mixed on bottle - Final volume was adjusted 250 ml.
Chloroform: Octanol	Chloroform Octanol	24:1 (v/v)	Chloroform and Isoamylalcohol mixed with 24:1 ratio of volume.
RNAse A	RNAse A	10mg/ml	Stock RNAse were diluted by the ratio of 1:10

2.3. Quantification and Qualification Assay of Genomic DNA

Concentrations of genomic DNA samples were measured by the help of spectrofotometer principle and Qubit protocol (**Figure 2.3**). Qubit 2.0Fluorometer and Qubit dsDNA (double stranded) BR Assay Kit were used in protocol (**Figure 2.4**). The reagent bound directly to DNA under dark conditions and make measurable to DNA amount.

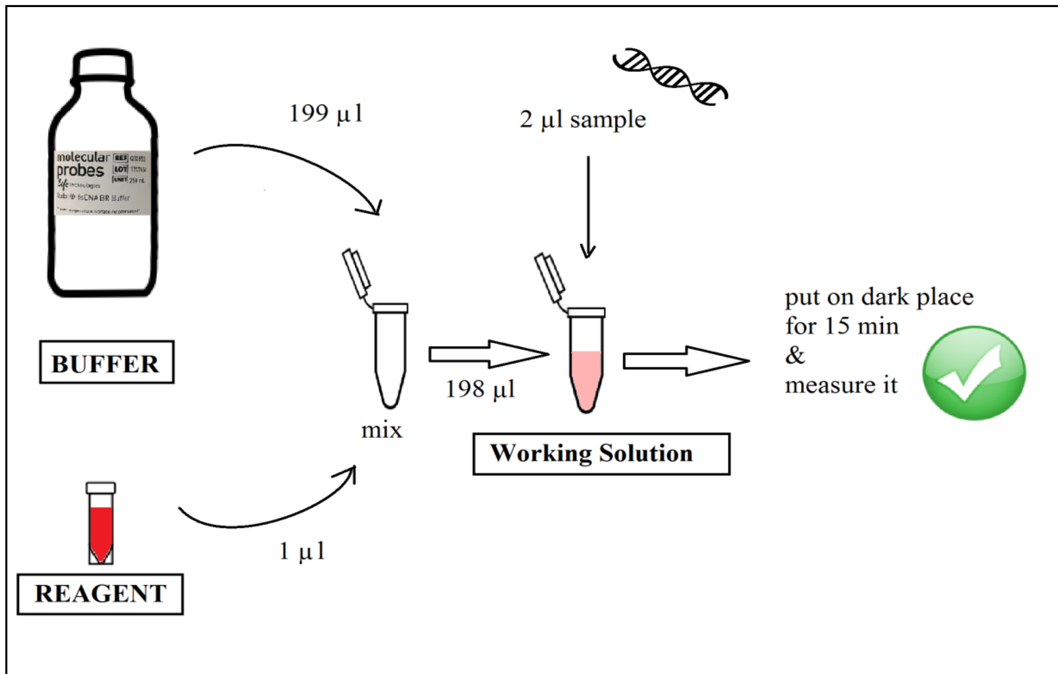

Figure 2.3. Qubit assay protocol

Figure 2.4. Qubit 2.0 Fluorometer and Qubit dsDNA BR Assay Kit (Buffer & Reagent)

Working solution for this measurement was prepared by mixing Qubit dsDNA BR reagent and Qubit dsDNA BR buffer solution in 1:200 proportions (1 µL reagent and 199 µL buffer solution) for each sample (Figure 2.2). 198 µL of the working solution is and 2 µL DNA sample was transferred on Qubit Assay tube. Obtained 200 µL mix was vortexed for a short time and incubated in a dark place. After 15 min incubation in dark, concentrations of the samples were measured by using Qubit 2.0 Fluorometer device.

For qualification analysis, genomic DNA samples were loaded on 0.8 % agarose gel and after 45 min running on electrophoresis system at 100 voltages.

Then genomic DNA samples were diluted to 2 ng/µl and stock &diluted genomic DNA samples were stored separately at -20 °C.

2.4. KASP (Competitive Allele Specific PCR)

Sex discrimination regions and their SNP positions are chosen from the study which is done by Kafkas in 2015. On that study 38 putative sex- associated SNP loci were determined in 28 RAD (Restriction site-associated DNA) reads among 33,757 polymorphic SNP markers. Initially 4 SNP position were selected according to their HRM (High Resolution Melting) positive results, but only 2of them (133396-167992) were defined as suitable for KASP primer transformation. For transform the KASP primer 50 base should be known before and after of SNP and there shouldn't be any other SNP near up to 30 nucleotides of the designated SNP points. One more translatable SNP (176863) was found in the same article, and total of 3 positions (**Figure 2.5**) were used in KASP Assay.

On experiment design; before the adjustment of device for Competitive Allele Specific PCR, reaction solutions were prepared on dark condition. Two component of KASP assay is Assay Mix and Master Mix **(Figure 2.6).** While Assay mix is varying according to the primer sequence, Master Mix containing the PCR reaction components such as reaction buffer, Taq polymerase enzyme, dNTPs and magnesium which are ready to use on KASP kit **(Figure 2.7)**. Assay Mix contains two forward primers (competitive primers) and one common reverse primer. These are specific for polymorphic part of genome. Master Mix also contains FRET cassettes involve fluorescent dye molecules (FAM/VIC™ and HEX dyes) together with PCR components.

No	Marker name	SNPs	Positions	Sequence (5′-3′)
1	SNP-PIS-1319	Y-Y	28-77	AATTCGTATAGCCCGTGAGAATACATG(Y)GGATGAGGGCTATACATGAGAGAGAGTACACT CACATGGCCAAGGGTT(Y)CGAAT
2	SNP-PIS-29689	Y-R	33-71	AATTCAACATCTTATAAAGCGAAATCACTTCA(Y)AATAATGCTTCTTCTTTGCAAGTGCACCA AACAATAT(R)TTGAATGATGA
3	SNP-PIS-112277	Y-R	10-61	AATTCGTTA(Y)CTAGAGGGTGATTTTAAAACTCTTACAGACACAAAACCATGACAATAATT(R) AAGGAAGAAAATTCAGCATGC
4	SNP-PIS-120693	M-W	19-57	AATTCAATGATCTAGATT(M)AAAGAAGGCATTGGATGTTGTGTATTGTCATTTGTAA(W)AAT ATCTTGGTGTGTAAAATGTGTA
5	SNP-PIS-127343	S	27	AATTCACCAATATTTTACTGCAATAA(S)TAAGAATGTAATGACAGGGTGAGTGAAAATG GTAGATTAAAATTTTAAGGAAATG
6	SNP-PIS-133396	S-Y	46-47	AATTCTCCTCTGTTTTTTGGGCAAACCGCAAAGAAGATTAAAGTA(S)(Y)TGATCCATGATCT TCAAGTTTCAGTACTATTCATA
7	SNP-PIS-135862	Y-Y	40-41	AATTCTTTGGTTTTGTGTCTGAATGTGGATAATATATGG(Y)(Y)GCCTCATGTTGATTATGGG AAATGTGCATGGAAATAGTATC
8	SNP-PIS-136404	K	23	AATTCTTTTAGGGGTTGTCAAA(K)TGACCGGATTCCTCACAAATTCAATTGCCAACTCT AAAGCTGGCAAGAAATTCTTTAGC
9	SNP-PIS-167992	W	36	AATTCAAACGAAAAATAACTTCATAGCGTGAGCTC(W)TTGTTCCACCTSTAACCGCAACC CTAAGCTGCAATTGATCACTTCC
10	SNP-PIS-174431	M	56	AATTCCATTACTTCAACAAGTCTCTAGCCGCGTACATATAAAAATTAACTACTCA(M)AGTGA AAGTGGAYAAATTGTTAAGTT
11	SNP-PIS-176863	R	57	AATTCCACATTTGACMAGGGTTGGAACTTTTGAGGTGGATGTGAGCTTGGAAGGTA(R) TATCATACTTTGCAGACAGGTCGAT

Figure 2.5. Selected SNP positions from Kafkas 2015 article.

FRET cassettes are affected from light so the Master Mix containing the FRET cassette should be protected from the light to inhibit unwanted irradiation. For this purpose, aluminium folio was enough to cover 2ml eppendorf tubes after aliquation of Master Mix stock.

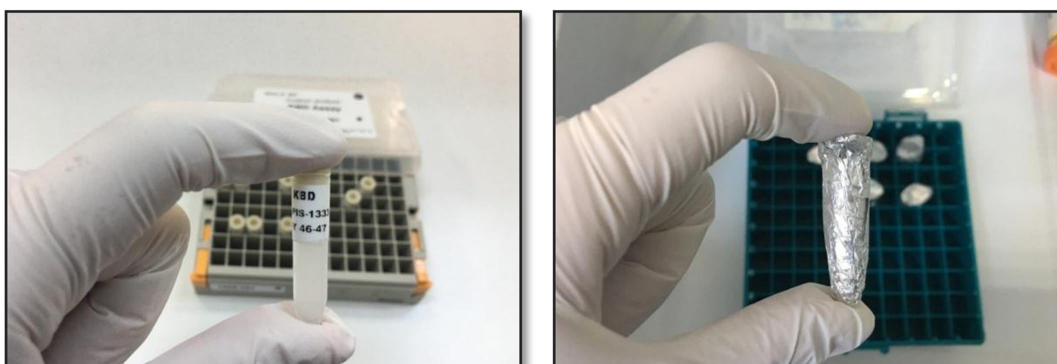

Figure 2.6. Assay mix (left) and Master mix (right)

After decision of sample and primer set number, required amount of mixture was prepared. Before the mixed solution 10µl 2ng concentrated DNA templates were put on wells. For each primer set; 0.28 µl Assay Mix (72x) and 10 Master Mix (2x) were combined on other clean tube than mixture aliquoted to MicroAmp® Optical 96-Well Plates' decided wells as 10.28 µl(**Figure 2.8**). Optic Adhesive Covers were used to seal

the plate for protection and reading radiation interface after reaction. Plate was centrifuged at 1000 rpm for 10 seconds to fall down of samples. Then, the plate is placed into machine. Applied Biosystems® StepOnePlus ™ Real Time PCR Systems was used as an instrument to perform the KASP Assay.

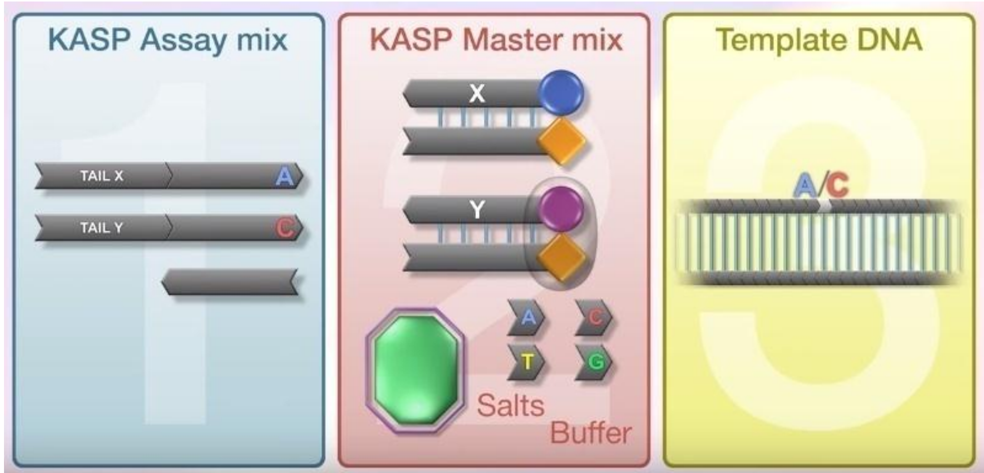

Figure 2.7. Solution contents which use in KASP assay

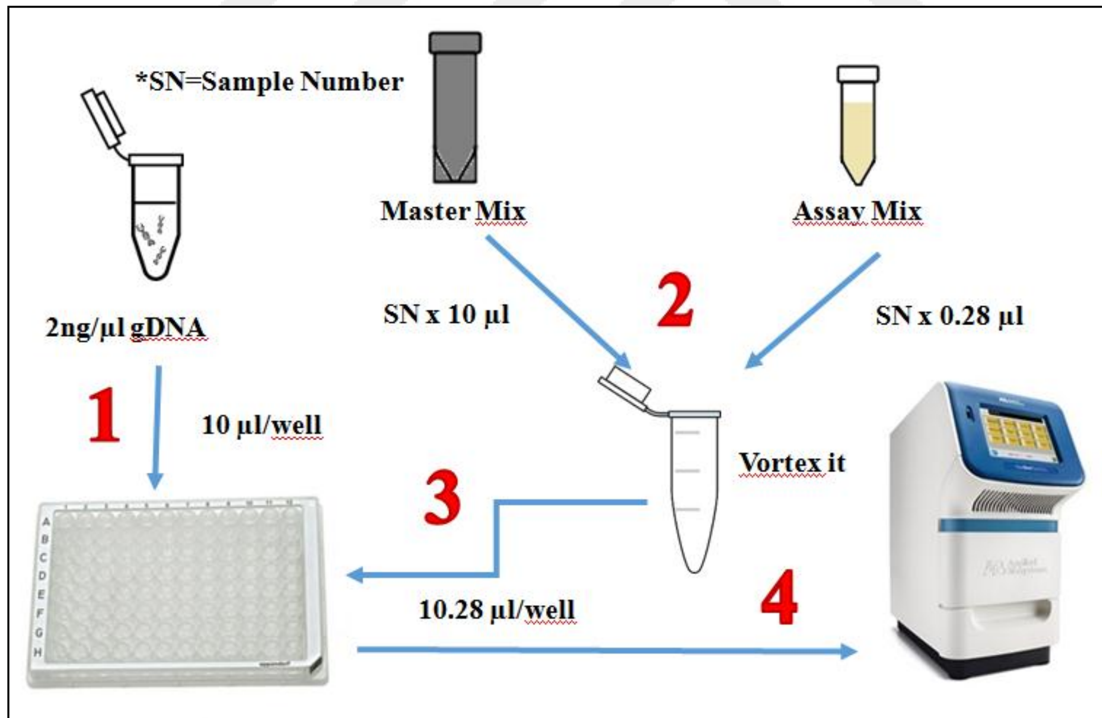

Figure 2.8. Schematic diagram of preparing KASP assay

Applied Biosystems' StepOne™ Software version 2.2.2 was used for experiment configuration. After choosing "Design Wizard" tab (**Figure 2.9**), specific experiment name was written. 96 well plate system was used which relevant with machine apparatus. Than "Genotyping" type of experiment was selected to detect of SNP variants on target nucleic acid sequence in sample. On the next page, as a reagent for genotyping "Other" were marked that means KASP reagents. "Wet DNA" was preferred as a template. On the instrument run section; "Standard" ramp speed, Pre-PCR read and Amplification stages were included to run. SNP assay properties were set manually. Allele1 was appointed to FAM reporter and Allele 2 was appointed to HEX (VIC™) reporter than NFQ-MGB (3' nonfluorescent quencher- minor groove binder) quencher was selected for both of them.

Figure 2.9. Program setup steps on Applied Biosystems' StepOne software

Sample and replicate numbers were entered and each primer minimum 2 negative control and none positive control were defined. The sample's places were leave as they are to organize after design by the "Edit Plate Layout" section. Reaction volume was entered 21μl. **Table 2.3** was applied as a run method.

Table 2.3. Temperature flow of KASP

Step	Stage 1	Stage 2	Stage 3 (10 cycles)		Stage 4 (26 cycles)		Read	Hold
Temperature	30.0°C	94°C	94°C	61°C	94°C	57°C	30.0°C	4°C
Time (min)	01:00	15:00	00:20	01:00	00:20	01:00	01:00	∞

After the thermocycling is over, the program shows pages which include Allelic Discrimination Plot **(Figure 2.10)** and correlative Plate Layout **(Figure 2.11)** side-by-side. Each dot on the plot demonstrate one well's signal results and according to received frequency of signal on well, dot place and colour appointed automatically by program. When user clicks the well/dot, connected dot/well beams (**Figure 2.10-11**). If Allele 2 (horizontal value) is high and Allele 1's value is almost zero, program colours the dark blue to dot that means, its homozygous for Allele 2 (**Figure 2.10**).If Allele 1 (vertical value) is high and Allele 2's value is almost zero, program colours a red to dot that means, its homozygous for Allele 1. If dot colour is green, the signals are received from both Alleles' dye and the sample is heterozygous. Also black square spots show the negative control and if they are not so close to zero there might be an error on experiment because there is no DNA on negative controls and reaction is not expected.

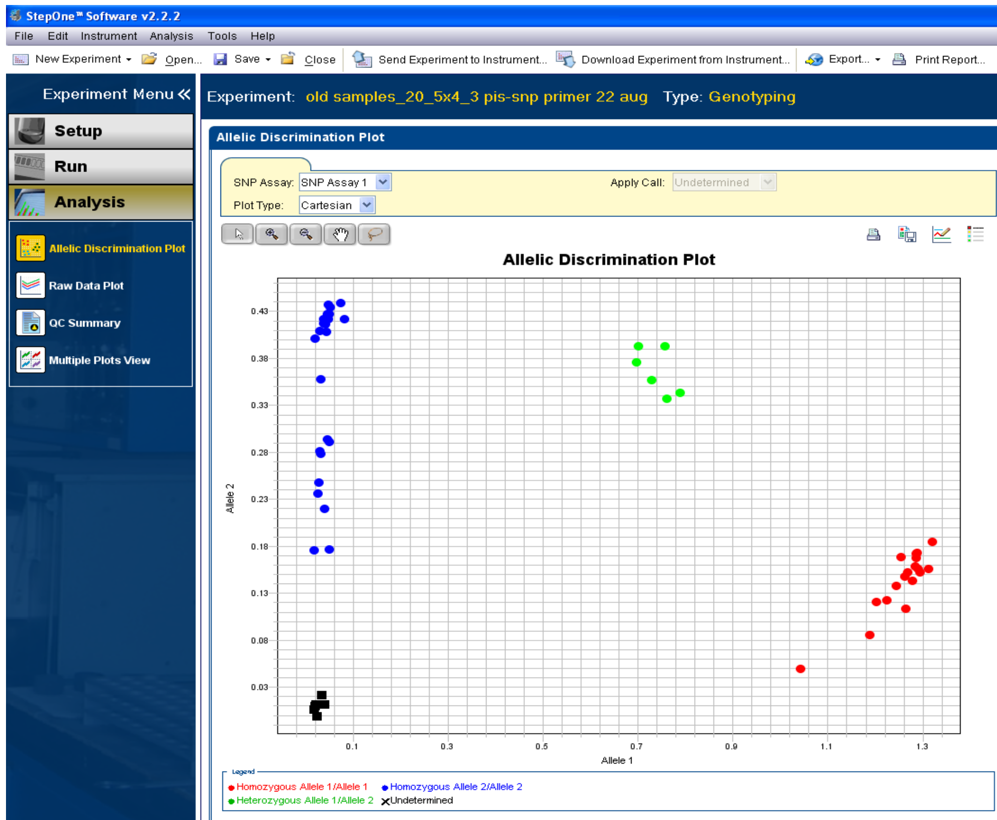

Figure 2.10. Allelic Discrimination Plot on StepOne Software (Screenshot)

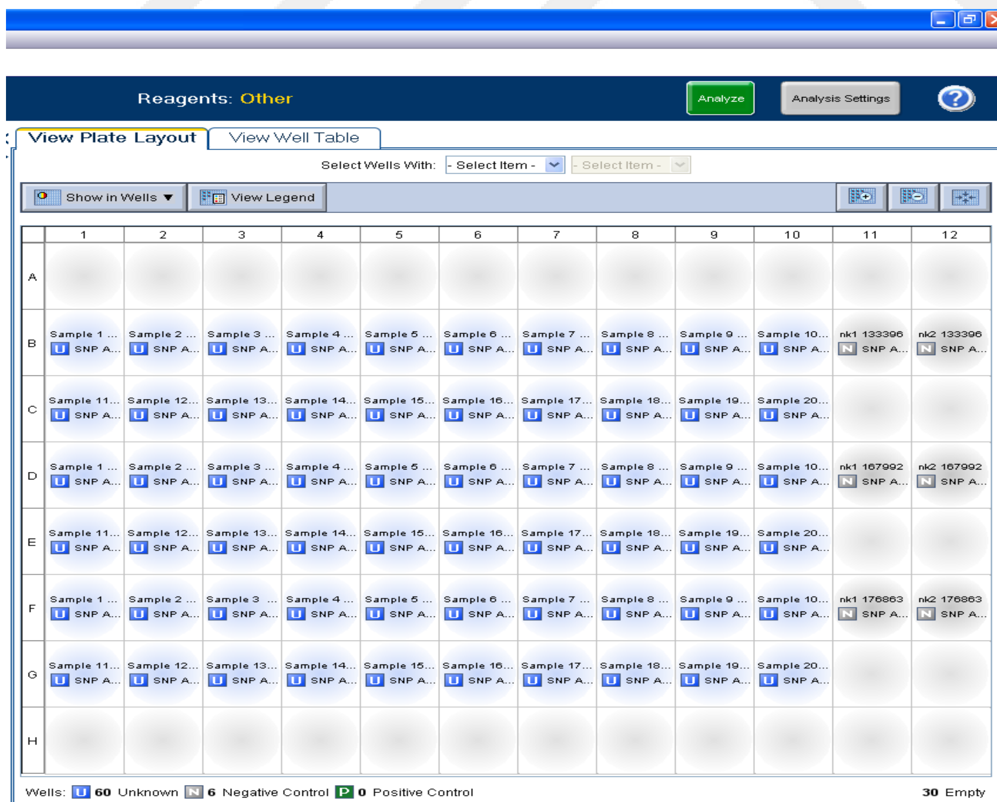

Figure 2.11. Plate Layout of on StepOne Software (Screenshot)

3. RESULTS and DISCUSSION

3.1. Quantification and Qualification Results of Isolated Genomic DNA

Doyle and Doyle (1991) procedures were used for DNA isolation. *Pistacia* contains highly phenolic compounds and these similar size and charge polyphenols known as major inhibitors of enzymatic activity during amplification (Schrader et al., 2012). Once the plant cells are broken apart, polyphenols become exposed to oxygen and reacted with polyphenol oxidases. Polyphenol oxidation products covalently bind to the phosphate backbone of nucleic acids, making them forcefully impossible to be removed (Borse et al., 2011).

Because of all these, it is important to use PVP (PolyVinylPyrrolidone) on DNA isolation procedure. Before the procedure which is given in Material Method section of thesis (Doyle and Doyle), Song&Herry (1995) isolation procedure was carried out but asit seems in the image of the gel (**Figure 3.1(a)**), obtained DNA's were not enough pure, which is why we think it will be phenolic. Then the procedure (Abuduli, 2015) which includes PVP to remove phenolic compounds was applied. Although the DNA concentrations were high (almost 470 ng/µl) after the procedure, smear image (**Figure 3.1 (b)**) was obtained, which meant broken DNA. Finally, the last version of procedure (given in Material Method part) was tried and desired results (**Figure 3.1 (d)**) were obtained to proceed to next step. Also chloroform & octanol step is repeated on procedure to obtain clarified DNA. Because the chloroform solution allows separation of the two phases, while the hydrophilic upper phase carry DNA and RNA, cell components like polypeptides, lipids and polysaccharides remain in hydrophobic lower layer. If this separation is not occurred sharply or any component from the lower phase mix to hydrophilic layer while upper phase transfer to clean tube; obtained DNA couldn't be very pure.

To understand the RNase's activity that we used, 2 different gDNA sample of *Pistacia* were loaded on %0.8 agarose gel after isolation, with before and after RNase implementations. As shown in **Figure 3.1 (d)**, uniform genomic DNAs were placed in the 4th and 5th wells, although there was a large RNA collection in the lower parts of the first two wells. That means our RNase worked very well.

On the next step by the help of Qubit DNA BR Assay Kit concentration of samples were determined **(Table 3.1)**. 100 µl 2 ng/µl DNA's were prepared on 0.5 ml tube by diluting from each sample on as shown in **Table 3.1** concentrations for KASP assay.

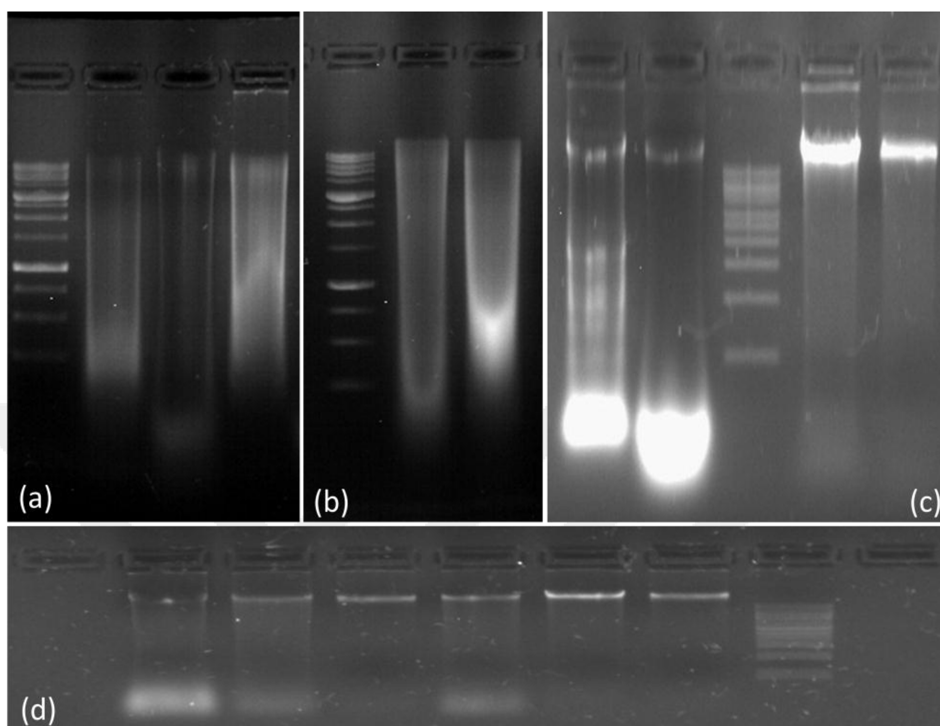

Figure 3.1. Agarose Gel (%0.8) image of Genomic DNA's after (a) Song & Herry (1995) isolation protocol, (b) Abuduli 2015 protocol, (c) First and secound wells are DNAs without RNA'ase; 4th and 5 th well are with RNA'ase implemantation 3th well is 1kb ladder.(d) Randomly selected 6 *pistacia*'s gDNA

Table 3.1. Genomic DNA concentrations of samples & required DNA and nucleic acid free H_2O to dilute 2ng/µl.

For 2ng/µl, 100 µl sample	**First conconcatration (ng/ µl)**	**DNA(µl)**	**H_20(µl)**
1. P. *atlantica* (Male)	81,4	2,46	97,54
2. P. *integerrima* (Male)	2,30	86,96	13,04
3. P. *khinjuk* (Male)	5,61	35,65	64,35
4. P. *mutica* (Male)	31,4	6,37	93,63

Table 3.1 (**continued**)

For 2ng/µl, 100 µl sample	First conconcatration (ng/ µl)	DNA(µl)	H_20(µl)
5. P. *palaestina* (Male)	6,36	31,45	68,55
6. P. *terebinthus* (Male)	24,4	8,20	91,80
7. P. *vera* (Male)	91,4	2,19	97,81
8. P. *lentiscus* L. (Male)	18,20	10,99	89,01
9. P. *atlantica* (Female)	3,20	62,50	37,50
10. P. *integerrima* (Female)	10,2	19,60	80,40
11. P. *khinjuk* (Female)	4,80	41,67	58,33
12. P. *mutica* (Female)	14,5	13,79	86,21
13. P. *palaestina* (Female)	6,23	32,10	67,90
14. P. *terebinthus* (Female)	10,1	19,80	80,20
15. P. *vera* (Female)	4,90	40,82	59,18
16. P. *lentiscus* L. (Female)	8,29	24,13	75,87

3.2. Results of KASP

In this study 3 SNP position was converted to KASP primer. These are SNP-PIS-133396, SNP-PIS- 167992 and SNP-PIS-176863. It is necessary to a few points to design a KASP primer from SNP points. Firstly, 50 base should be known before and after of SNP. Then there shouldn't be any other SNP near up to 30 nucleotides of the designated SNP points. Three primers actualized these conditions.

For each primer; sequence of primers, partner fluorescent dye, related SNP nucleotide and GC contents of primers decided as shown in **Table 2.2-4.** SNP nucleotides are end point of primers for forward primer couples. The signals are created by the help of fluorescent partner which are FAM or HEX at the end of thermo cycler. These dyes create signal when environment temperature at 30 °C so if this reading stage doesn't include to flow diagram there wouldn't be any signal to read. In our assay this reading step was 1 minute. After reading, system automatically creates a plot for each well.

Table 3.2. Data's about SNP-PIS-133396 primer.

SNP-PIS-133396	Primer Sequences (5' – 3')	Allele	CG %
Primer FAM	GCAAACCGCAAAGAAGATTAAAGTAC	[C]	38,5
Primer HEX	GCAAACCGCAAAGAAGATTAAAGTAG	[G]	38,5
Common Primer	GAATAGTACTGAAACTTGAAGATCATG GAT		33,3
Amplifying Sequence	AATTCTCCTCTGTTTTTGGGCAAACCGCAAAGAAGATTAAAG TA[C/G]YTGATCCATGATCTTCAAGTTTCAGTACTATTCATA		

Table 3.3. Data's about SNP-PIS-167992 primer.

SNP-PIS-167992	Primer Sequences (5' – 3')	Allele	CG %
Primer FAM	GAAAAATAACTTCATAGCGTGAGCTCA	[A]	37
Primer HEX	GAAAAATAACTTCATAGCGTGAGCTCT	[T]	37
Common Primer	TTAGGGTTGCGGTTASAGGTGGAA		50
Amplifying Sequence	AATTCAAACGAAAAATAACTTCATAGCGTGAGCTC[A/T]TTGT TCCACCTSTAACCGCAACCCTAAGCTGCAATTGATCACTTCC		

Table 3.4. Data's about SNP-PIS-176863 primer.

SNP-PIS-176863	Primer Sequences (3'–5')	Allele	CG %
Primer FAM	ATCGACCTGTCTGCAAAGTATGATAT	[A]	38,5
Primer HEX	CGACCTGTCTGCAAAGTATGATAC	[G]	44
Common Primer	AGGTGGATGTGAGCTTGGAAGGTA		50

Amplifying Sequence	AATTCCACATTTGACMAGGGTTGGAACTTTTGAGGTGGATGTG AGCTTGGAAGGTA[A/G]TATCATACTTTGCAGACAGGTCGAT

On the 133396 (C/G) SNP position KASP results **(Figure 3.2)**, *Pistacia vera* female (green dot) showed as a heterozygous character and Allele 1&2 signal frequencies were 0,6776 & 0,3324 respectively (green highlighted), while all other male and female species (dark blue dots) have homozygous Allele 2 character (**Figure 3.2, Table 3.5**). That means *Pistacia vera* female individual is carry both Cytosine and Guanine on different alleles while *Pistacia vera* male carry just Cytosine on its both alleles. Also this result was supportive to *Pistacia*'s ZW/ZZ (female/male) Sex Determination System as reported (Kafkas, 2015). Negative control (black squares dots) signals are almost "0", so solutions are not contaminated.

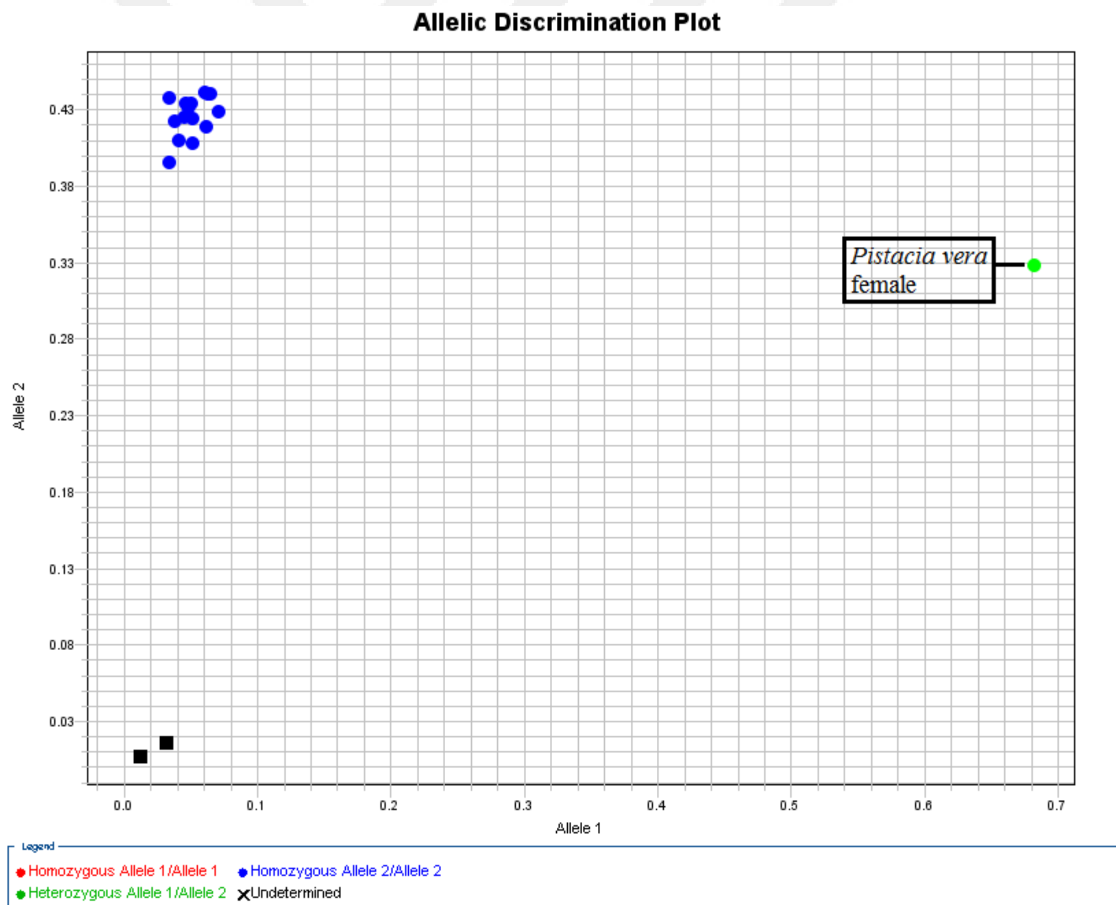

Figure 3.2. Allelic discrimination plot obtained from 133396 primer results.

Table 3.5. Allelic frequencies of species for SNP 133396

Species Name	Allel 1 Signal Frequency (ΔRn)	Allel 2 Signal Frequency (ΔRn)
● *P. atlantica* Male	0,0287	0,3993
● *P. integerrima* Male	0,0461	0,4125
● *P. khinjuk* Male	0,0453	0,4384
● *P. mutica* Male	0,0358	0,4144
● *P. palaestina*Male	0,0400	0,4290
● *P. terebinthus* Male	0,0417	0,4379
● *P.vera* Male	0,0467	0,4287
● *P. lentiscus* Male	0,0293	0,4423
● *P. atlantica* Female	0,0434	0,4355
● *P. integerrima*Female	0,0600	0,4442
● *P. khinjuk* Female	0,0580	0,4446
● *P. mutica* Female	0,0659	0,4325
● *P. palaestina* Female	0,0553	0,4453
● *P. terebinthus* Female	0,0334	0,4265
● *P. vera* Female	0,6776	0,3324
● *P. lentiscus* Female	0,0563	0,4229
■ NK 133396-1	0,0273	0,0205
■ NK 133396-2	0,0071	0,0113

On the 167992 (A/T) SNP position KASP results **(Figure 3.3)**, *Pistacia atlantica* female, *Pistacia vera* female and *Pistacia terebinthus* female (green dots) showed as a heterozygous character. Allele 1&2 signal frequencies were 0,2281 & 0,1638 for *P. atlantica*; 0,1666 & 0,1498 for *P. vera*; 0,2773 & 0,2132 *for P. terebinthus* respectively. The males of these 3 (*atlantica, vera, terebinthus*) female species showed homozygous Allele 2 character (dark blue dots) like all other male and female species (**Figure 3.3, Table 3.6**). Furthermore, this is a promising result because SNP positions are already determined by *Pistacia vera* species but this result also showed that SNP 167992 was

succeed for 2 more species (*atlantica and terebinthus*).Solutions are not contaminated because negative control dots are placing appropriate positions.

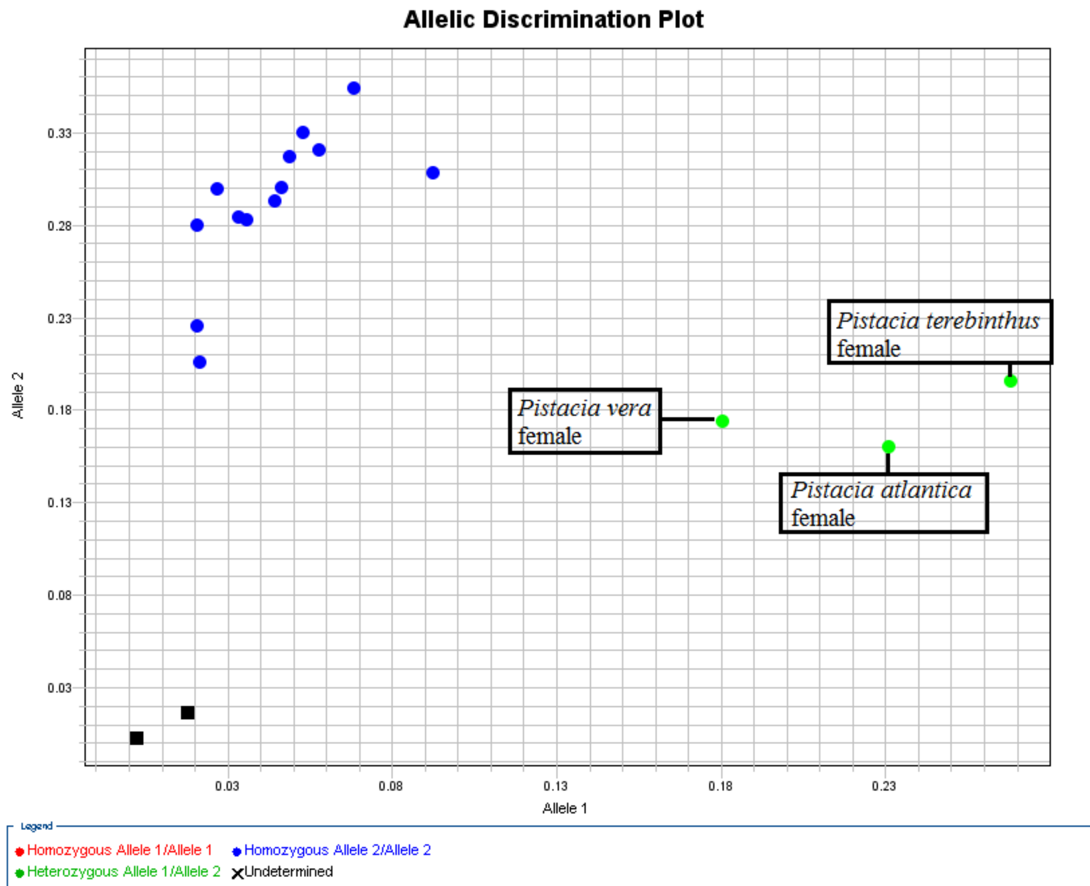

Figure 3.3. Allelic discrimination plot obtained from 167992 primer results.

Table 3.6. Allelic frequencies of species for SNP 167992

Species Name	Allele 1 Signal Frequency(ΔRn)	Allele 2 Signal Frequency(ΔRn)
● *P. atlantica* Male	0,0340	0,2979
● *P. integerrima* Male	0,0687	0,3337
● *P. khinjuk* Male	0,0631	0,3330
● *P. mutica* Male	0,0601	0,2785
● *P. palaestina* Male	0,0775	0,3556
● *P. terebinthus* Male	0,0608	0,3371
● *P.vera* Male	0,0440	0,3138

● *P. lentiscus* Male	0,0385	0,2539
●*P. atlantica* Female	0,2281	0,1638
● *P. integerrima* Female	0,0455	0,3329
● *P. khinjuk* Female	0,0499	0,3326
● *P. mutica* Female	0,0951	0,3143
● *P. palaestina* Female	0,0669	0,2940
●*P. terebinthus* Female	0,2773	0,2132
●*P. vera* Female	0,1666	0,1498
● *P. lentiscus* Female	0,0102	0,2445
■ NK 167992-1	0,0178	0,0208
■ NK167992-2	0,0118	0,0179

In the analysis of of SNP 176863 (A/G) marker, any polymorphism was not seen between species or sexes. All samples showed highly homozygous Allele 1 character (red dots) **(Figure 3.4).** While at the first sight dot position shown on the diagonal line, allele 1 signal frequencies range of samples are between 1,1563 and 1,3647 **(Table 3.7),**allele 2 signals are maximum 0,23 ΔRn. It may conclude that SNP 176863 marker is not distinctive for gender or among species, despite our designed primer is worked properly on PCR reaction. It is unsurprising that, due to their bi-allelic nature, not all SNPs will be polymorphic for all species.

Luminance is a photometric measure of the luminous intensity per unit area (cd/m^2) of light travelling in a given direction. Each primer showed different luminance frequency ranges depending on length and sequence factors. All of the results showed that the frequency ranges of the 3 primers we screened had similar frequency ranges with the study performed with similar condition in our laboratory before (Kösoğlu et al., 2017). On that study, researchers analysed 6 SNP markers associated with Pl_{arg}, $Pl_{13}$and Pl_8 resistance genes in sunflower for downy mildew. Three parental crosses and F_2 individuals were examined with KASP analysis. This study was a reliable small-scale breeding study, in addition to showing similarities with our implementations and homozygote/heterozygout discrimination, in our study the KASP assay has also been shown an application power in terms of gender discrimination as a new field.

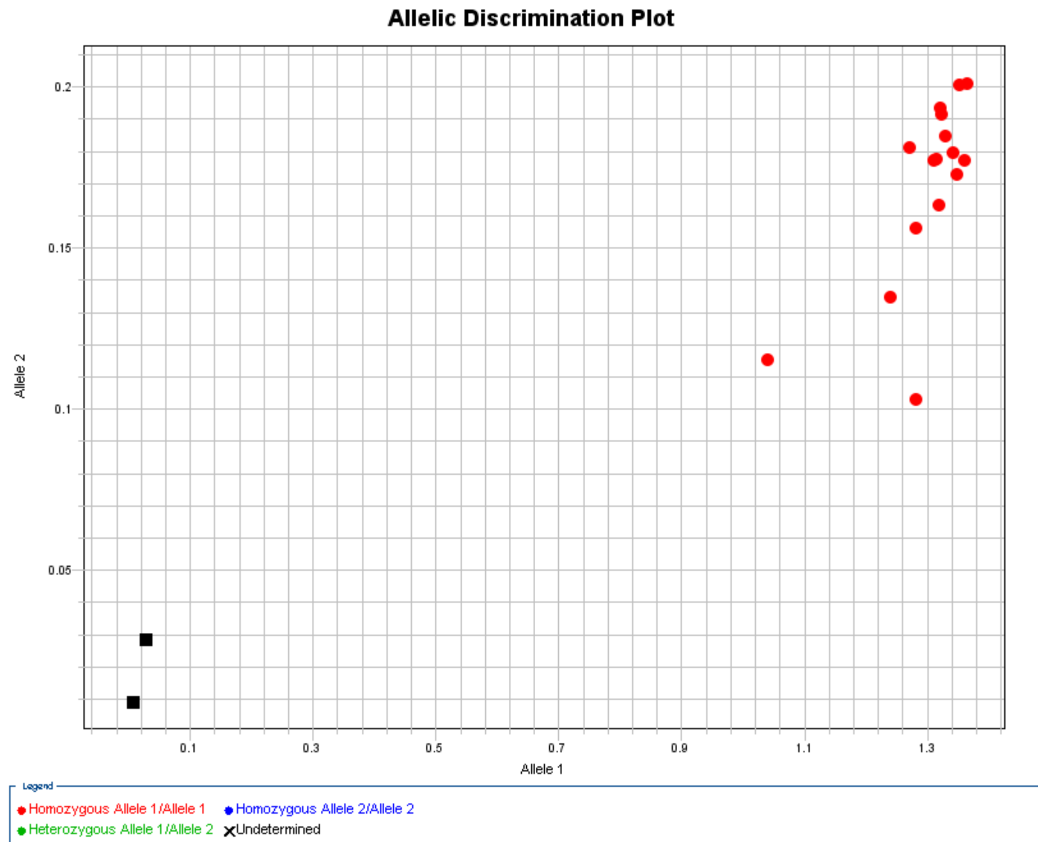

Figure 3.4. Allelic discrimination plot obtained from 176863 primer results.

In this research, it's realized that there is no enough genome data and marker discovery related to the *Pistacia* genus. Moreover, not just for sex discrimination, there are just several molecular marker studies even for other most significant character which is highly adaptable to drought and salty. One of the marker approaches relevant to salinity-related gene was done by Jazi et al. (2017). On that study first whole transcriptome survey was completed and it made a way for functional and comparative genomic studies about pistachio (*Pistacia vera* L.) cultivars.

It's obviously known that, this new and popular technique KASP assay has a wide range of usage on different livings from crop improvements (Semagn et al., 2014) to detection of disease in human (Landoulsi et al., 2017). Nonetheless, sex discrimination studies using KASP technique is not seen except for a few fish like salmon (Carmichael et al., 2013) or halibut (Palaiokostas et al., 2013) and one insect species (De Boer et al., 2015). These results show that, this thesis study is a not just first for *Pistacia* in terms of gender discrimination but also the precursor study for sex discrimination by KASP in plants.

Table 3.7. Allelic frequencies of species for SNP 176863

Species Name	Allele 1 Signal Frequency(ΔRn)	Allele 2 Signal Frequency(ΔRn)
● *P. atlantica* Male	1,2726	0,1964
● *P. integerrima* Male	1,3565	0,2143
● *P. khinjuk* Male	1,3565	0,2065
● *P. mutica* Male	1,2576	0,1548
● *P. palaestina* Male	1,3114	0,2040
● *P. terebinthus* Male	1,3402	0,1952
● *P. vera* Male	1,2477	0,1696
● *P. lentiscus* Male	1,2145	0,0998
● *P. atlantica* Female	1,3056	0,1929
● *P. integerrima* Female	1,3511	0,2333
● *P. khinjuk* Female	1,3534	0,2307
● *P. mutica* Female	1,3421	0,2011
● *P. palaestina* Female	1,3647	0,2348
● *P. terebinthus* Female	1,3375	0,2106
● *P. vera* Female	1,1563	0,1696
● *P. lentiscus* Female	1,2389	0,1466
■ NK 176863-1	0,0068	0,0193
■ NK 176863-2	0,0019	0,0098

There are also sex discrimination marker studies already done for the *Pistacia* species and different molecular marker methods have been used in these studies like RAPD (Esfendiyari et al., 2012) or ISSR (Ehsanpour et al., 2008). When the previous studies were compared with the results of KASP; there are extra steps in previous techniques like preparing gel, loading samples or screening the gel. These extra steps can cause disadvantageous in terms of time, effort and cost. While each extra step increases the risk of making mistakes, KASP is very suitable for scale-up as it does not contain any of these steps.

4. CONCLUSIONS

In this thesis study; the aim is to find molecular markers depending on the single nucleotide difference between the sexes of the species in the *Pistacia* genus. For this purpose, KASP assay was selected as a fastest and most accurate technology while clearly isolated genomic DNAs was used as a template DNA. The leaves of male and female individuals were collected for eight different *Pistacia* species. Among these species, *Pistacia vera* L's fruit and *Pistacia lentiscus* L's resin were of particular importance for our country and for the worldwide economy.

The results were evaluated on the basis of the frequency values after KASP assay. The 167992 (A/T) SNP discriminated the 3 female individuals of *Pistacia* which are *P. atlantica* Desf, *P. vera* L. and *P. terebinthus* L., 133396 (C/G) SNP primer discriminated only *P. vera* L. female and any polymorphism was not seen for SNP 176863 (A/G) position.

The single nucleotide differences can affect the sequences of amino acids and accordingly protein structure. One protein can change whole pathway of organism's function. These SNP's are found in genome abundantly that means plenty of chance to ascertain the meaningful one. In this research we focused on gender differences of the genus because this difference affects many features of the plants and, additionally, using molecular techniques may help to screen the sex of plant seedlings so quickly. Especially pistachio producers are still use morphological data to understand the gender of individual and while these characters like leaf or body size may change according to external factors, the formation of sex organs like staminate or pistillate takes over years.

This study showed potential use of SNP markers in *Pistacia* with KASP assay. The results of the study can be used in breeding programs focused on sex discrimination. As a next step of this study; especially for the *Pistacia vera* which is the most economically prevalent and important *Pistacia* species, could be collected from the very different regions and found markers had verified.

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Project Assignment

Marmara University Research Foundation (Project No: FEN-C-YLP-090217-0049); Ekim 2014-Haziran 2015.